Uniwersytet Rolniczy im. H. Kołłątaja w Krakowie
Wydział Biotechnologii i Ogrodnictwa

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Nr albumu: 1054

Analiza porównawcza dzikich taksonów z rodzaju *Daucus* L. na podstawie danych cytogenetycznych oraz wybranych cech morfologicznych i anatomicznych

Praca doktorska

Praca wykonana pod kierunkiem
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Składam serdeczne podziękowania

dr hab. inż. Ewie Grzebelus, prof. URK

za ogromne wsparcie, otwartość, życzliwość, zaufanie, wyrozumiałość,
cenne uwagi merytoryczne oraz za te kilka lat owocnej współpracy

Praca powstała w ramach realizacji projektu badawczego PRELUDIUM 18 pod tytułem „Powiązania ewolucyjne w rodzaju *Daucus*: cytogenetyczna i morfo-anatomiczna analiza porównawcza dzikich krewniaków marchwi” finansowanego ze środków Narodowego Centrum Nauki (2020–2023).

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Kierownik projektu: mgr inż. Dariusz Kadłuczka

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Tytuł projektu: „Porównawcza analiza dystrybucji chromosomowej sekwencji repetytywnych u wybranych gatunków podrodziny Apioideae”

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Ministerstwo Nauki
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Spis treści

Wykaz publikacji wchodzących w skład rozprawy doktorskiej

Publikacja 1 (P1):

Kadluczka D.✉, Grzebelus E.✉ 2021. Using carrot centromeric repeats to study karyotype relationships in the genus *Daucus* (Apiaceae). *BMC Genomics*, 22, 508.
DOI: https://doi.org/10.1186/s12864-021-07853-2

Punktacja MEiN*$_{2021}$: **140**
IF**$_{2021}$: **4,558**

Publikacja 2 (P2):

Kadluczka D.✉, Sliwinska E., Grzebelus E.✉ 2022. Combining genome size and pollen morphology data to study species relationships in the genus *Daucus* (Apiaceae). *BMC Plant Biology*, 22, 382.
DOI: https://doi.org/10.1186/s12870-022-03743-1

Punktacja MEiN$_{2021}$: **140**
IF$_{2021}$: **5,260**

Publikacja 3 (P3):

Kadluczka D., Grzebelus E.✉ 2022. Comparative fruit morphology and anatomy of wild relatives of carrot (*Daucus*, Apiaceae). *Agriculture*, 12, 2104.
DOI: https://doi.org/10.3390/agriculture12122104

Punktacja MEiN$_{2021}$: **100**
IF$_{2021}$: **3,408**

Sumaryczna punktacja MEiN: 380
Sumaryczny IF: 13,226

✉ – autor korespondencyjny

* Punktacja MEiN – liczba punktów przyznanych za publikację w czasopiśmie na podstawie komunikatu Ministra Edukacji i Nauki z dnia 21 grudnia 2021 r.

** IF (ang. *Impact Factor*) – współczynnik wpływu dla czasopisma (zgodnie z rokiem opublikowania)

Pozostały dorobek naukowy

Wykaz publikacji niebędących przedmiotem rozprawy doktorskiej

1. Kwiatkowska M., **Kadłuczka D.**, Wędzony M., Dedicova B., Grzebelus E. 2019. Refinement of a clearing protocol to study crassinucellate ovules of the sugar beet (*Beta vulgaris* L., Amaranthaceae). *Plant Methods*, 15, 71.
   DOI: https://doi.org/10.1186/s13007-019-0452-6

   Punktacja MNiSW*$_{2019}$: **140**

   IF$_{2019}$: **3,610**

2. Grzebelus D., Macko-Podgórni A., Stelmach K., Kwolek K., **Kadłuczka D.**, Gajewski Z., Barański R. 2019. Opracowanie i wykorzystanie wysokowydajnych technik selekcji genomowej w doskonaleniu warzyw. *Biuletyn Instytutu Hodowli i Aklimatyzacji Roślin*, 286, 313–317.
   DOI: https://doi.org/10.37317/biul-2019-0070

   Punktacja MNiSW$_{2019}$: **20**

3. Klimek-Chodacka M., **Kadluczka D.**, Lukasiewicz A., Malec-Pala A., Baranski R., Grzebelus E. 2020. Effective callus induction and plant regeneration in callus and protoplast cultures of *Nigella damascena* L. *Plant Cell Tissue and Organ Culture*, 143, 693–707.
   DOI: https://doi.org/10.1007/s11240-020-01953-9

   Punktacja MNiSW$_{2019}$: **100**

   IF$_{2020}$: **2,711**

4. **Kadluczka D.**, Czernicka M., Sliwinska E., Bieniasz M, Maćkowska K, Kapczyńska A., Grzebelus E. 2021. Development and quality of pollen in *Lachenalia* cultivars with determination of genome size and chromosome number. *Scientia Horticulturae*, 277, 109842.
   DOI: https://doi.org/10.1016/j.scienta.2020.109842

   Punktacja MEiN$_{2021}$: **140**

   IF$_{2021}$: **4,342**

* Punktacja MNiSW – liczba punktów przyznanych za publikację w czasopiśmie na podstawie komunikatu Ministra Nauki i Szkolnictwa Wyższego z dnia 18 grudnia 2019 r.

Wykaz doniesień konferencyjnych

1. **Kadłuczka D.**, Macko-Podgórni A., Grzebelus E. Analiza porównawcza wybranych sekwencji repetytywnych na chromosomach w rodzaju *Daucus* z wykorzystaniem fluorescencyjnej hybrydyzacji *in situ*. *IV Ogólnopolska Konferencja Doktorantów Nauk o Życiu – BioOpen,* 24–25.05.2018, Łódź, Polska.

   (Konferencja krajowa – referat)

2. **Kadłuczka D.**, Macko-Podgórni A., Iovene M., Grzebelus E. Comparative analysis of selected repetitive sequences on chromosomes in the genus *Daucus* using fluorescence *in situ* hybridization (FISH). *2nd ISHS International Symposium on Carrots and other Apiaceae,* 19–22.09.2018, Kraków, Polska.

   (Konferencja międzynarodowa – poster)

3. **Kadłuczka D.**, Kapczyńska A., Czernicka M., Bieniasz M., Maćkowska K., Grzebelus E. Comparative morphology and viability of pollen in some *Lachenalia* cultivars. *8th International Conference for Young Researchers "Multidirectional Research in Agriculture, Forest and Technology"*, 25–27.04.2019, Kraków, Polska.

   (Konferencja międzynarodowa – referat)

4. Kwiatkowska M., **Kadłuczka D.**, Wędzony M., Dedicova B., Grzebelus E. Optymalizacja techniki przejaśniania gruboośrodkowych zalążków buraka cukrowego (*Beta vulgaris* L., Amaranthaceae) salicylanem metylu. *58 Zjazd Polskiego Towarzystwa Botanicznego "Botanika bez granic"*, 1–7.07.2019, Kraków, Polska.

   (Konferencja krajowa – poster)

5. Grzebelus E., Malec-Pala A., **Kadłuczka D.**, Morańska E., Oleszkiewicz T. Regeneration ability in protoplast cultures of the cultivated Apiaceae. *10th PSEPB Conference "Experimental plant biology at various scales: from molecules to the environment"*, 20–23.09.2021, Katowice, Polska.

   (Konferencja międzynarodowa – poster)

6. **Kadłuczka D.**, Grzebelus E. Mapowanie porównawcze powtórzeń centromerowych pochodzących z marchwi u wybranych taksonów z rodzaju *Daucus* (Apiaceae). *„Genetyka aplikacyjna roślin – wyzwania XXI wieku"*, 22–24.09.2021, Warszawa, Polska.

   (Konferencja krajowa – poster)

7. **Kadłuczka D.**, Grzebelus E. Comparative FISH mapping of carrot-derived centromeric repeats in *Daucus* (Apiaceae). *Mendel Genetics Conference*, 20–23.07.2022, Brno, Czechy.

   (Konferencja międzynarodowa – poster)

Udział w projektach badawczych

- **2022**

  Tytuł projektu: „Wykorzystanie somatycznej hybrydyzacji do poszerzenia zakresu zmienności wybranych roślin warzywnych”
  Źródło finansowania: Ministerstwo Rolnictwa i Rozwoju Wsi (badania podstawowe na rzecz postępu biologicznego w produkcji roślinnej)

  Numer projektu: DHR.hn.802.13.2022

  Charakter udziału w projekcie: wykonawca

- **2020–2023**

  Tytuł projektu: „Powiązania ewolucyjne w rodzaju *Daucus*: cytogenetyczna i morfo-anatomiczna analiza porównawcza dzikich krewniaków marchwi”

  Źródło finansowania: Narodowe Centrum Nauki (PRELUDIUM 18)

  Numer projektu: UMO-2019/35/N/NZ9/00959

  Charakter udziału w projekcie: kierownik projektu

- **2019**

  Tytuł projektu: „Transfer cytoplazmatycznej męskiej sterylności poprzez somatyczną hybrydyzację u marchwi”

  Źródło finansowania: Ministerstwo Rolnictwa i Rozwoju Wsi (badania podstawowe na rzecz postępu biologicznego w produkcji roślinnej)

  Numer projektu: HOR-hn-801-PB-5/16-9

  Charakter udziału w projekcie: wykonawca

- **2018–2019**

  Tytuł projektu: „Porównawcza analiza dystrybucji chromosomowej sekwencji repetytywnych u wybranych gatunków podrodziny Apioideae”

  Źródło finansowania: Ministerstwo Nauki i Szkolnictwa Wyższego (dotacja celowa na prowadzenie badań naukowych lub prac rozwojowych oraz zadań z nimi związanych, służących rozwojowi młodych naukowców oraz uczestników studiów doktoranckich)

  Charakter udziału w projekcie: kierownik projektu

- **2017–2018**
  Tytuł projektu: „Histological evaluation of sugar beet ovules”

  Źródło finansowania: Syngenta Seeds AB, Szwecja (badania zamawiane)

  Numer projektu: 928/WBiO/IBRiB/17-18

  Charakter udziału w projekcie: wykonawca

Doświadczenie naukowe zdobyte za granicą

- **1.11.2021–31.01.2022** (3 miesiące)
  Technische Universität Dresden, Faculty of Biology, Institute of Botany, Drezno, Niemcy – staż w ramach programu Erasmus+
- **1.10–31.12.2018** (3 miesiące)
  National Research Council of Italy, Institute of Biosciences and Bioresources, Portici, Włochy – staż w ramach programu Erasmus+

Liczbowe zestawienie dorobku naukowego (na dzień 22.03.2023)

Rodzaj publikacji	**N**	**Punktacja MNiSW/MEiN**	**IF**	**Liczba cytowań[1]**
Publikacje w czasopismach znajdujących się w bazie *Journal Citation Reports* (JCR)	6	760	23,889	20
Publikacje w czasopismach innych niż znajdujące się w bazie JCR	1	20	–	–
Razem		**780**	**23,889**	**20**
Indeks Hirscha (*h*-index)			**3**	

[1] według bazy *Scopus*

Wykaz stosowanych skrótów

BAC sztuczny chromosom bakteryjny (ang. *bacterial artificial chromosome*)

DCRE powtórzenie specyficzne dla marchwi (ang. *carrot-specific repetitive element*)

FAO Organizacja Narodów Zjednoczonych do spraw Wyżywienia i Rolnictwa (ang. *Food and Agriculture Organization of the United Nations*)

FISH fluorescencyjna hybrydyzacja *in situ* (ang. *fluorescence in situ hybridization*)

HOR powtórzenie wyższego rzędu (ang. *higher-order repeat*)

Mpz milion par zasad

NOR obszar jąderkotwórczy, organizator jąderka (ang. *nucleolar organizing region*)

RAPD losowa amplifikacja polimorficznych fragmentów DNA (ang. *random amplified polymorphic DNA*)

SEM skaningowa mikroskopia elektronowa (ang. *scanning electron microscopy*)

1. Streszczenie

Aby sprostać wzrastającemu zapotrzebowaniu na żywność oraz zapewnić bezpieczeństwo żywnościowe dla stale rosnącej populacji ludności, hodowcy wymagają dostępu do nowych zasobów genetycznych, które mogłyby być wykorzystane w programach hodowlanych w celu poszerzenia zmienności genetycznej roślin uprawnych. W tym kontekście dzikie gatunki rodzaju *Daucus* L. mogłyby odegrać ważną rolę w procesie doskonalenia współczesnego rolnictwa, stanowiąc potencjalne źródło genów istotnych z punktu widzenia hodowli roślin.

Rodzaj *Daucus* stanowi doskonały model do badań porównawczych. Obejmuje marchew uprawną (*D. carota* subsp. *sativus* Hoffm.) – podgatunek o zsekwencjonowanym genomie – oraz około 40 dzikich gatunków, które różnią się pod względem genetycznym i morfologicznym. Jednakże powiązania taksonomiczne i filogenetyczne w obrębie tego rodzaju nie zostały jeszcze w całości wyjaśnione, a ponadto dane cytogenetyczne i morfologiczne dla jego przedstawicieli są wciąż niepełne.

W ramach prezentowanej rozprawy doktorskiej przeprowadzono szereg badań ukierunkowanych na bliższe poznanie rodzaju *Daucus*. Ich celem było: (1) porównawcze mapowanie cytogenetyczne sekwencji powtarzalnej CentDc (zidentyfikowanej w genomie marchwi uprawnej) u wybranych taksonów rodzaju *Daucus* i gatunków spokrewnionych za pomocą fluorescencyjnej hybrydyzacji *in situ* (FISH), (2) określenie zawartości jądrowego DNA w ich genomach, a także (3) porównanie morfologii ich pyłku oraz morfologii i anatomii ich owoców.

Porównawcza analiza FISH wykazała obecność powtórzeń CentDc w genomach 26 obiektów (reprezentujących 15 taksonów) rodzaju *Daucus* i jednego gatunku spokrewnionego. W przypadku 20 obiektów *Daucus* (11 taksonów) sonda CentDc hybrydyzowała do obszarów centromerowych wszystkich chromosomów tych obiektów. Pozostałe FISH-pozytywne gatunki wykazywały wzór hybrydyzacyjny zróżnicowany pod względem liczby par chromosomów zawierających powtórzenia CentDc. Obecność tych powtórzeń w genomach dzikich krewniaków marchwi sugeruje, że sekwencja ta występowała w genomie ich wspólnego przodka.

Analiza cytometryczna wykazała 3,2-krotne zróżnicowanie zawartości jądrowego DNA wśród taksonów rodzaju *Daucus*, która mieściła się w zakresie od 0,999 do 3,228 pg. Znaczne różnice w wielkości genomu zaobserwowane u dzikich gatunków rodzaju *Daucus* sugerują, że w trakcie specjacji w ich genomach wystąpiły duże rearanżacje chromosomalne i/lub nagromadzenie powtarzalnych sekwencji DNA.

Analiza palinologiczna wykazała, że pyłek badanych taksonów różni się pod względem wielkości i kształtu. W przypadku *Daucus* średnia długość osi biegunowej ziaren pyłku mieściła się w zakresie od 21,19 do 40,38 µm. Wszystkie ziarna pyłku cechowała obecność trzech porusów, natomiast pod względem urzeźbienia egzyny wyróżniono kilka typów morfologicznych: prążkowany, pomarszczony, perforowany oraz typy mieszane.

W przypadku owoców badanych taksonów zaobserwowano szeroki zakres różnic dotyczących ich cech morfo-anatomicznych. W rodzaju *Daucus* obserwowane różnice dotyczyły wielkości owoców (2,1–8,4 mm) i ich kształtu (od jajowatego do podłużnego), a także urzeźbienia powierzchni owoców oraz ich niektórych cech anatomicznych.

Uzyskane wyniki mogą pomóc w wyjaśnianiu powiązań taksonomicznych między gatunkami rodzaju *Daucus* oraz w prawidłowej identyfikacji zasobów genowych zgromadzonych w bankach genów, a w szerszej perspektywie – mogą przyczynić się do rozwoju przyszłych programów hodowlanych marchwi uprawnej.

2. Summary

To address the rising need for food and to ensure food security for a constantly growing population, plant breeders require access to new genetic resources that could be used in breeding programs to expand the genetic variation of crops. In this context, wild *Daucus* L. species may play a crucial role in the process of improving modern agriculture, being a potential source of genes important from a plant breeding perspective.

The genus *Daucus* is an excellent model for comparative studies. It contains the cultivated carrot (*D. carota* subsp. *sativus* Hoffm.) – a subspecies with a sequenced genome – and about 40 wild species which differ genetically and morphologically. However, the taxonomic and phylogenetic relationships among *Daucus* have not yet been fully clarified, and moreover, cytogenetic and morphological data for its members are still incomplete.

In this doctoral dissertation, several studies focusing on a better understanding of the genus *Daucus* were performed. The aims of these studies were (1) to cytogenetically map the CentDc repetitive sequence (identified in the carrot genome) in selected *Daucus* taxa and related species using fluorescence *in situ* hybridization (FISH), (2) to estimate the nuclear DNA content in their genomes, and (3) to compare the pollen morphology as well as the fruit morphology and anatomy of these taxa.

Comparative FISH analysis revealed the presence of the CentDc repeats in the genomes of 26 *Daucus* accessions (representing 15 taxa) and one closely related species. In the case of 20 *Daucus* accessions (representing 11 taxa), the CentDc probe hybridized to the centromeric regions of all chromosomes of these accessions. The other FISH-positive species displayed different hybridization patterns that varied in terms of the number of chromosome pairs with the CentDc repeats. The presence of these repeats in the genomes of wild relatives of carrot suggests that this sequence was present in the genome of their common ancestor.

The flow cytometric analysis showed a 3.2-fold variation in the nuclear DNA content among *Daucus* taxa, ranging from 0.999 to 3.228 pg. The great differences in the genome size observed in wild *Daucus* species suggest that in the course of speciation, large-scale chromosomal rearrangements or the accumulation of repetitive DNA sequences occurred in their genomes.

The palynological analysis showed that the pollen of the studied taxa differed in size and shape. In *Daucus*, the mean length of the pollen polar axis varied from 21.19 to 40.38 μm. All pollen grains were tricolporate, while in terms of exine ornamentation, several morphological types were distinguished: striate, rugulate, perforate, and mixed types.

In the case of fruits of the studied taxa, a wide range of variations in their morpho-anatomical characteristics was observed. For *Daucus*, the observed differences included the fruit size (2.1–8.4 mm) and shape (from ellipsoid to oblong), as well as the fruit surface sculpturing and some anatomical characteristics.

These findings may help in elucidating the taxonomic relationships among *Daucus* species and in the correct identification of gene bank accessions. In a broader perspective, they can contribute to the development of future carrot breeding programs.

3. Wprowadzenie

Rodzaj *Daucus* L. należy do rodziny Apiaceae (selerowate) zawierającej 466 rodzajów i 3820 gatunków szeroko rozpowszechnionych na świecie, występujących zwłaszcza w strefach umiarkowanych półkuli północnej (Plunkett i in. 2019). Z ekonomicznego punktu widzenia Apiaceae jest jedną z najważniejszych rodzin roślin okrytonasiennych, która obejmuje nie tylko marchew uprawną (*D. carota* subsp. *sativus* Hoffm.), ale też liczne inne warzywa korzeniowe oraz zioła i przyprawy, takie jak pasternak zwyczajny (*Pastinaca sativa* L.), pietruszkę zwyczajną [*Petroselinum crispum* (Mill.) Fuss], seler zwyczajny (*Apium graveolens* L.), koper ogrodowy (*Anethum graveolens* L.), lubczyk ogrodowy (*Levisticum officinale* W.D.J. Koch), fenkuł włoski (*Foeniculum vulgare* Mill.), kmin rzymski (*Cuminum cyminum* L.), kminek zwyczajny (*Carum carvi* L.) czy kolendrę siewną (*Coriandrum sativum* L.) (Rubatzky i in. 1999). Cechą charakterystyczną tych roślin jest obecność substancji zapachowych należących do grupy metabolitów wtórnych (olejki eteryczne), stąd też są powszechnie wykorzystywane w celach kulinarnych i medycynie niekonwencjonalnej (Rubatzky i Yamaguchi 1997; Pollastro i Gaeta 2020).

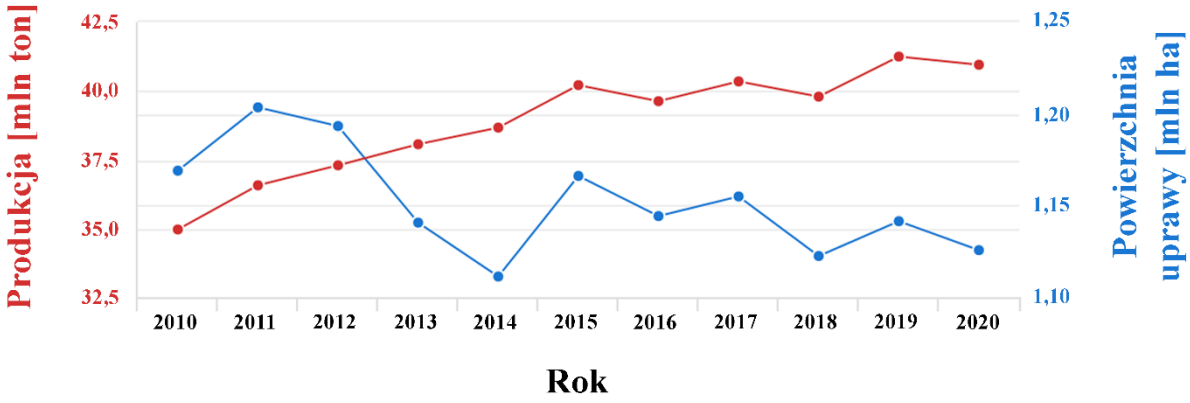
3.1. Rodzaj *Daucus*

3.1.1. Marchew uprawna i dzikie gatunki

Pod względem ekonomicznym i żywieniowym marchew uprawna jest najważniejszym przedstawicielem rodzaju *Daucus*. Wśród warzyw gruntowych zajmuje drugie miejsce pod względem powierzchni uprawy i zbiorów w Polsce. Według danych Głównego Urzędu Statystycznego powierzchnia uprawy marchwi w roku 2019 wyniosła 22,2 tys. ha, a zbiory – 679 tys. ton (Rocznik Statystyczny Rolnictwa 2021). Z kolei w rankingu globalnym, według Organizacji Narodów Zjednoczonych do spraw Wyżywienia i Rolnictwa (ang. *Food and Agriculture Organization of the United Nations,* FAO), marchew znajduje się w pierwszej dziesiątce najważniejszych warzyw – jej światowa produkcja w roku 2020 wyniosła 40,95 mln ton (**Rycina 1**) (FAO 2022). Należy jednak zwrócić uwagę, że FAO gromadzi te dane wspólnie dla marchwi i rzepy, ale wartości dla tej pierwszej stanowią tu ponad 95% (Simon 2019).

Przyjmuje się, że marchew została udomowiona około 1100 lat temu, a za najbardziej prawdopodobne miejsce jej udomowienia uważa się Azję Środkową (Iorizzo i in. 2013). Pierwsze udomowione rośliny marchwi wytwarzały korzenie o barwie żółtej i fioletowej. Następnie rozprzestrzeniły się one na zachód od Azji Centralnej i dotarły do Syrii, Afryki

Północnej, regionów basenu Morza Śródziemnego i Europy Południowej (XI–XIV wiek), a także na wschód – do Chin, Indii i Japonii (XIII–XVII wiek) (Banga 1963; Simon 2000). Marchew o pomarańczowym korzeniu pojawiła się dopiero w XVI wieku na terenie Niderlandów i powstała prawdopodobnie na skutek mutacji żółtej formy, a następnie selekcji przez człowieka (wtórne udomowienie) (Stolarczyk i Janick 2011; Iorizzo i in. 2013).



Rycina 1. Produkcja (–•–) i powierzchnia uprawy (–•–) marchwi i rzepy na świecie w latach 2010–2020 (FAO 2022)

Zmiana preferencji z żółtych form marchwi na pomarańczowe i ich rozpowszechnienie przez hodowców miały ogromne znaczenie dla dzisiejszych konsumentów, ponieważ marchew stała się głównym źródłem prowitaminy A (α- i β-karoten) w diecie człowieka (Heinonen 1990; Simon i in. 2008; Khoo i in. 2011). Dalsze działania hodowlane przyczyniły się do znacznego zwiększenia jej wartości odżywczej; dla przykładu, w Stanach Zjednoczonych zawartość karotenów w korzeniu marchwi jest obecnie o 50% wyższa niż w przypadku odmian uprawianych jeszcze pięć dekad temu (Simon i in. 2009). Karotenoidy są odpowiedzialne za żółtą (luteina), pomarańczową (α- i β-karoten) i czerwoną (likopen) barwę korzenia, natomiast kolor fioletowy pochodzi od antocyjanów należących do grupy związków polifenolowych (Arscott i Tanumihardjo 2010). Wszystkie te pigmenty wykazują właściwości prozdrowotne, między innymi wspomagają układ odpornościowy, zmniejszają ryzyko powstawania niektórych nowotworów, zapobiegają chorobom układu krążenia i chorobom wzroku (Lin i in. 2016; Manayi i in. 2016; Rowles i in. 2017; Langi i in. 2018; Chen i in. 2021; Hussain i in. 2022). Wysoka zawartość karotenoidów w korzeniu marchwi i ich znaczenie żywieniowe sprawiły, że roślina ta stała się doskonałym modelem do badań nad biosyntezą i akumulacją tych związków (Just i in. 2007; Iorizzo i in. 2016; Ellison i in. 2017; Ma i in. 2017; Coe i in. 2021; Oleszkiewicz i in. 2021ab; Zhao i in. 2022; Singh i in. 2023).

Dzikie gatunki *Daucus* są szeroko rozpowszechnione w strefach umiarkowanych półkuli północnej, ale najliczniej występują w regionie śródziemnomorskim, który uznaje się za centrum zróżnicowania tego rodzaju; kilka gatunków można również spotkać w Ameryce Południowej, Australii, a także w tropikalnej części Afryki (Grzebelus i in. 2011; Spooner 2019). Są to przede wszystkim dwuletnie rośliny zielne, rzadziej jednoroczne (Grzebelus i in. 2011; Plunkett i in. 2019), ale występuje też kilka gatunków drzewiastych – endemicznych dla wysp Makaronezji (Frankiewicz i in. 2020).

Pomimo ogromnych wysiłków badawczych podjętych w ciągu ostatnich kilku dekad, powiązania taksonomiczne i filogenetyczne w obrębie rodzaju *Daucus* nie zostały jeszcze w pełni wyjaśnione. Początkowo – na podstawie cech morfologicznych i anatomicznych – Sáenz Laín (1981) skatalogował 20 gatunków i podzielił je na pięć sekcji: *Daucus* L., *Anisactis* DC., *Platyspermum* DC., *Chrysodaucus* Thell i *Meoides* Lange. Następnie Rubatzky i in. (1999) rozszerzyli tę klasyfikację do 25 gatunków. Do lepszego zrozumienia powiązań filogenetycznych między gatunkami rodzaju *Daucus* i ich bliskimi krewniakami z podrodziny Apioideae przyczyniły się badania molekularne wykorzystujące różnorodne zestawy danych sekwencyjnych, w tym markery chloroplastowe (m.in. *rbc*L, *mat*K, *rpl*16, *rps*16, *rpo*C1, *trn*H-*psb*A, *rpo*B-*trn*C) i mitochondrialne, wewnętrzne sekwencje transkrybowane (ang. *internal transcribed spacer*, ITS) w obrębie sekwencji kodujących rRNA, markery jądrowe oparte na ortologach genów, markery polimorfizmu pojedynczego nukleotydu (ang. *single nucleotide polymorphism*, SNP) oraz całkowite sekwencje chloroplastowe i mitochondrialne (Downie i Katz-Downie 1996, 1999; Plunkett i in. 1996ab; Downie i in. 2000, 2001, 2010; Spalik i Downie 2007; Zhou i in. 2009; Spooner i in. 2013, 2017, 2020; Arbizu i in. 2014, 2016ab; Weitzel i in. 2014; Downie i Jansen 2015; Banasiak i in. 2016; Clarkson i in. 2021; Samigullin i in. 2022). Wyniki tych badań doprowadziły do podzielenia rodzaju *Daucus* na dwa główne klady: *Daucus* I i *Daucus* II, a także – dzięki pracom Banasiaka i in. (2016) – do jego rozszerzenia poprzez włączenie do *Daucus* rodzajów *Agrocharis* Hochst. (cztery gatunki), *Melanoselinum* Hoffm. (jeden gatunek), *Monizia* Lowe (jeden gatunek), *Pachyctenium* Maire et Pamp. (jeden gatunek), *Pseudorlaya* (Murb.) Murb. (dwa gatunki), *Rouya* Coincy (jeden gatunek), *Tornabenea* Parl. (sześć gatunków) oraz gatunków *Athamanta dellacellae* E.A. Durand et Barratte i *Cryptotaenia elegans* Webb ex Bolle; tym samym rodzaj *Daucus* obejmuje obecnie około 40 gatunków.

3.1.2. Genom i cytogenetyka

Genom marchwi uprawnej został niedawno zsekwencjonowany i dokładnie scharakteryzowany (Iorizzo i in. 2016). Do tego celu wykorzystano linię podwojonego haploida (ang. *doubled haploid*, DH1) marchwi typu nantejskiego o pomarańczowej barwie korzenia. Wielkość tego genomu oszacowano na 473 miliony par zasad (Mpz), co odpowiada wcześniejszym szacunkom opartym na analizach cytometrycznych (Arumuganathan i Earle 1991). Sekwencje powtarzalne (repetytywne) stanowią 46% (193,7 Mpz) genomu referencyjnego marchwi, z czego zdecydowana większość (97,9%) to ruchome elementy genetyczne – retrotranspozony (129,2 Mpz) i transpozony DNA (57,4 Mpz). Spośród tandemowych sekwencji repetytywnych wyróżnić można cztery główne ich rodziny: poznaną wcześniej sekwencję centromerową CentDc (CL1) (Iovene i in. 2011) oraz trzy nowe powtórzenia (CL8, CL80 i CL81). Z kolei liczba genów wynosi 32 113 (108,2 Mpz), z których 79% cechuje wysoka homologia do genów już opisanych (Iorizzo i in. 2016).

Haploidalna liczba chromosomów u gatunków należących do rodzaju *Daucus* mieści się w zakresie od n = 8 do 11 (Spooner 2019). Jedno z pierwszych doniesień o somatycznej liczbie chromosomów marchwi ($2n$ = 18) zostało opublikowane w latach 30. XX wieku (Lindenbein 1932). Dalsze badania cytotaksonomiczne potwierdziły, że zarówno uprawne, jak i dzikie formy *D. carota* są diploidami zawierającymi dziewięć par chromosomów (Sharma i Ghosh 1954; Sharma i Bhattacharyya 1959; Bell i Constance 1960). Taka liczba chromosomów występuje jeszcze u czterech innych gatunków: *D. annuus* i *D. insularis* – będących przedstawicielami niedawno włączonego do *Daucus* rodzaju *Tornabenea* – oraz *D. sahariensis* i *D. syrticus* (Grosso i in. 2008; Grzebelus i in. 2011). Jednak u znacznej większości gatunków tego rodzaju somatyczna liczba chromosomów wynosi $2n$ = 20 lub 22. Przedstawiciele *Daucus* to w przeważającej części diploidy, lecz występuje tu również kilka gatunków poliploidalnych: cztery tetraploidalne (*D. glochidiatus*, *D. incognitus*, *D. melananthos* i *D. pedunculatus*; $2n$ = 44) i jeden heksaploidalny (*D. montanus*; $2n$ = 66) (Iovene i in. 2008; Rice i in. 2015; Spooner 2019).

Chromosomy marchwi są małe (2–4 μm) i bardzo do siebie podobne, co uniemożliwia ich odróżnienie na podstawie wielkości i morfologii (Iovene i in. 2008; Nowicka i in. 2012). W przeszłości do identyfikacji chromosomów marchwi próbowano stosować podstawowe barwienia różnicowe, takie jak techniki barwienia prążkowego C oraz Q, ale te nie przyniosły w pełni zadowalających rezultatów (Kumar i Widholm 1984; Essad i Maunoury 1985). Nieco lepsze wyniki uzyskano, wykorzystując metodę barwienia prążków C na chromosomach

w stadium prometafazy (Schrader i in. 2003). Jednak dopiero zastosowanie fluorescencyjnej hybrydyzacji *in situ* (ang. *fluorescence in situ hybridization*, FISH) – jednej z najważniejszych technik cytogenetyki molekularnej – umożliwiło dokładniejszą analizę kariotypu marchwi i pozwoliło na identyfikację jej chromosomów (Iovene i Grzebelus 2019).

Utworzenie biblioteki klonów BAC (ang. *bacterial artificial chromosome* – sztuczny chromosom bakteryjny) dla marchwi (Cavagnaro i in. 2009) dało możliwość zastosowania metody FISH z wykorzystaniem wybranych – chromosomowo specyficznych – klonów BAC jako sond molekularnych, dzięki czemu udało się opisać standardowy kariotyp marchwi (Iovene i in. 2011). W tej samej pracy autorzy zidentyfikowali także sekwencję satelitarną, która hybrydyzowała do regionów centromerowych wszystkich chromosomów marchwi. Dalsze badania wykazały, że powtórzenie to (nazwane CentDc) zbudowane jest z monomerów o długości 159 pz, a każdy taki monomer składa się z czterech mniejszych – różniących się nieznacznie – podjednostek (A, B, C, D) o długości 39–40 pz, tworząc tzw. strukturę powtórzeń wyższego rzędu (ang. *higher-order repeat*, HOR) (Iovene i in. 2011; Iorizzo i in. 2016).

Poza klonami BAC inne typy sekwencji o pojedynczej lub niskiej liczbie kopii jako cytogenetyczne markery chromosomowo specyficzne nie były szeroko stosowane u marchwi. Ostatnio jednak Macko-Podgórni i in. (2017) wykorzystali poznany we wcześniejszych pracach marker *cult* różnicujący marchew dziką i uprawną (Grzebelus i in. 2014; Macko-Podgórni i in. 2014) i przeprowadzili jego mapowanie za pomocą FISH. W efekcie tej analizy wykazano, że marker *cult* znajduje się w części dystalnej długiego ramienia chromosomu 2 marchwi uprawnej. Ponadto w obrębie tego regionu autorzy zidentyfikowali gen *DcAHLc1* – potencjalnie związany z udomowieniem marchwi (gen kandydujący).

W ostatnich latach do badań nad chromosomami marchwi wykorzystywano również różne rodzaje sekwencji powtarzalnych. Nowicka i in. (2012) zastosowali system losowej amplifikacji polimorficznych fragmentów DNA (ang. *random amplified polymorphic DNA*, RAPD) i wybrali kilkanaście produktów amplifikacji (amplikonów) o długości od 517 do 1758 pz, które posłużyły do przygotowania sond do FISH. Cztery spośród tych sond dały wyraźny wzór hybrydyzacyjny na wszystkich lub prawie wszystkich chromosomach mitotycznych marchwi, przy czym większość sygnałów była zlokalizowana w regionach przycentromerowych i miała postać wzoru punktowego (ang. *dot-like*), co sugeruje, że sekwencje te są zorganizowane w klastry zawierające wiele kopii. Autorzy wykazali ponadto, że jednoczesne zastosowanie dwóch z tych czterech sond RAPD w połączeniu z sondą centromerową (CentDc) generuje specyficzny wzór hybrydyzacyjny umożliwiający identyfikację poszczególnych chromosomów marchwi.

W innej pracy ci sami autorzy (Nowicka i in. 2016a) zademonstrowali użyteczność innych sekwencji repetytywnych jako sond do FISH, mianowicie miniaturowych ruchomych elementów genetycznych (ang. *miniature inverted-repeat transposable elements*, MITEs) oraz zidentyfikowanych wcześniej powtórzeń specyficznych dla marchwi (ang. *carrot-specific repetitive elements*, DCREs; Cavagnaro i in. 2009). W przypadku tych pierwszych były to – poznane już w genomie marchwi – elementy *Krak* (Grzebelus i in. 2006; Grzebelus i Simon 2009) oraz *DcSto* (Macko-Podgorni i in. 2013), które jako sondy hybrydyzowały do wszystkich chromosomów marchwi, dając sygnały fluorescencyjne rozproszone na całej ich długości, z wyjątkiem obszaru jąderkotwórczego (ang. *nucleolar organizing region*, NOR) oraz regionów centromerowych i telomerowych. Jednakże w porównaniu z sondami *Krak* sondy *DcSto* generowały silniejsze sygnały o wzorze przypominającym prążki (ang. *banding-like pattern*), a ich intensywność różniła się pomiędzy poszczególnymi chromosomami, podczas gdy intensywność sygnałów *Krak* była w tym aspekcie względnie jednolita. Ponadto analiza FISH z użyciem sondy *DcSto*/*Krak* w połączeniu z sondami centromerową (CentDc) i telomerową umożliwiła autorom odróżnienie poszczególnych par chromosomów marchwi. Natomiast odnośnie do powtórzeń z grupy DCRE, spośród jedenastu sekwencji wybranych jako potencjalne markery chromosomowe trzy (DCRE9, DCRE16 i DCRE22) generowały specyficzny wzór hybrydyzacyjny na chromosomach marchwi, przy czym zarówno rozmieszczenie, jak i intensywność sygnałów znacznie się różniły w zależności od zastosowanej sondy. Ponadto intensywność sygnałów dla każdej z tych sond różniła się pomiędzy poszczególnymi chromosomami, co sugeruje, że zawierają one różną liczbę kopii powtórzeń DCRE. Ta różnica w sile sygnałów, a także jednoczesne zastosowanie sond centromerowej i telomerowej umożliwiły autorom dokonanie precyzyjnych pomiarów chromosomów marchwi oraz ich odróżnienie (Nowicka i in. 2016a).

Jedne z pierwszych porównawczych badań cytogenetycznych z użyciem FISH u dzikich gatunków rodzaju *Daucus* dotyczyły określenia fizycznej lokalizacji sekwencji kodujących rRNA – genów 5S i 18S–25S rDNA (Iovene i in. 2008). W pracy tej wykorzystano – oprócz kilku ważnych ekonomicznie taksonów z podrodziny Apioideae – osiem dzikich gatunków *Daucus* znajdujących się w różnej odległości filogenetycznej w stosunku do marchwi. Porównawcza analiza FISH wykazała, że gatunki rodzaju *Daucus*, pomimo różnej liczby chromosomów, nie różniły się liczbą *loci* genów rDNA, tj. u wszystkich obserwowano pojedyncze *loci* dla obu genów: 5S rDNA występował w jednej parze chromosomów, zaś 18S–25S rDNA – w przewężeniu wtórnym innej pary (NOR). W kolejnej pracy Iovene i in. (2011) wykorzystali wspomniane wcześniej klony BAC i wykonali mapowanie porównawcze

za pomocą FISH u dwóch dzikich gatunków marchwi należących do różnych kladów *Daucus*, mianowicie *D. crinitus* (*Daucus* I; 2*n* = 22) i *D. pusillus* (*Daucus* II; 2*n* = 22). W efekcie tych badań zidentyfikowano regiony synteniczne w genomach tych gatunków oraz zwrócono uwagę na obecność możliwych rearanżacji chromosomowych w rodzaju *Daucus*.

W kontekście zidentyfikowanych u marchwi powtórzeń centromerowych CentDc Iorizzo i in. (2016) wykorzystali dane sekwencyjne dla pięciu innych gatunków reprezentujących oba klady *Daucus* i przeprowadzili porównawczą analizę *in silico*, która wykazała, że powtórzenia podobne do CentDc stanowią przeważającą część tandemowych sekwencji repetytywnych w genomach *D. syrticus* (2*n* = 18; powtórzenie nazwane Ds-CL1) i *D. aureus* (2*n* = 22; Da-CL1), obu należących do tego samego kladu (*Daucus* I). Jednakże u *D. syrticus* powtórzenie to było zorganizowane w strukturze HOR, podobnie jak w przypadku sekwencji CentDc zidentyfikowanej u marchwi, natomiast u *D. aureus* powtórzenie Da-CL1 nie przejawiało cech takiej struktury – składało się bowiem z jednakowych podjednostek o długości 40 pz. W przypadku *D. pusillus* (2*n* = 22; *Daucus* II) jedynie początkowa podjednostka (40 pz) najliczniej występującej sekwencji repetytywnej w jego genomie (Dp-CL5) wykazywała wyraźne podobieństwo do podjednostki A oryginalnego powtórzenia CentDc marchwi. Wyniki te świadczą o tym, że rodziny powtórzeń satelitarnych CentDc, Ds-CL1, Da-CL1 i Dp-CL5 dzielą wspólne pochodzenie ewolucyjne. Natomiast u pozostałych gatunków – *D. guttatus* (2*n* = 20) i *D. littoralis* (2*n* = 20) należących do kladu *Daucus* II – nie zidentyfikowano sekwencji podobnych do CentDc.

Inną obficie występującą sekwencją satelitarną w rodzaju *Daucus* jest CL80 (Iorizzo i in. 2016). Powtórzenie to wykryto w genomach gatunków należących do *Daucus* I (marchew uprawna) i *Daucus* II (*D. guttatus* i *D. littoralis*), co sugeruje, że pochodzenie CL80 poprzedza dywergencję tych kladów. Sekwencja CL80 u badanych gatunków była konserwatywna (podobieństwo sekwencji powyżej 96%), lecz liczba jej kopii, a także – jak potwierdzono cytogenetycznie – lokalizacja chromosomowa różniły się między gatunkami. W przypadku marchwi sygnały FISH dla CL80 występowały tylko w pierwszej parze chromosomów, natomiast u *D. littoralis* sonda ta hybrydyzowała do wszystkich chromosomów, a u *D. guttatus* – do prawie wszystkich. Ponadto u tych dwóch ostatnich powtórzenia CL80 były najliczniej reprezentowaną rodziną sekwencji repetytywnych, stanowiąc odpowiednio 2,4 i 3,9% ich genomów.

Dane dotyczące zawartości jądrowego DNA w rodzaju *Daucus* są dostępne dla wielu dzikich gatunków i podgatunków oraz licznych odmian/linii marchwi uprawnej i ukazują duże zróżnicowanie tego parametru, przyjmując wartości od 0,847 do 3,019 pg/2C DNA (Bai i in.

2012; Pustahija i in. 2013; Tavares i in. 2014; Nowicka i in. 2016b; Roxo i in. 2021). Średnia zawartość DNA u marchwi uprawnej wynosi 0,96 pg/2C DNA, co wykazały najszersze jak dotąd badania Nowickiej i in. (2016b), w których do analizy cytometrycznej wykorzystano znaczną liczbę roślin reprezentujących aż 26 odmian/linii marchwi o różnym zabarwieniu korzenia. W przypadku badanych w tej samej pracy 14 dzikich podgatunków *D. carota* ($2n = 18$) – zawartość 2C DNA mieściła się w przedziale od 0,9 do 1,1 pg, natomiast w obrębie dziewięciu dzikich diploidalnych gatunków *Daucus* autorzy zaobserwowali ponadtrzykrotną różnicę w zawartości 2C DNA, stwierdzając jednocześnie brak zależności między tym parametrem a liczbą chromosomów u tych gatunków.

3.2. Uzasadnienie podjęcia tematu badawczego

Aby sprostać wzrastającemu zapotrzebowaniu na żywność oraz zapewnić bezpieczeństwo żywnościowe dla stale rosnącej populacji ludności, hodowcy wymagają dostępu do nowych zasobów genetycznych, które mogłyby być wykorzystane w programach hodowlanych w celu poszerzenia utraconej w procesie udomowienia i hodowli zmienności genetycznej roślin uprawnych. Konieczność poszukiwania nowych źródeł tej zmienności jest o tyle kluczowym wyzwaniem, że różnorodność świata roślinnego stoi w obliczu zagrożeń związanych z postępującymi zmianami klimatu i działalnością człowieka. Tak zróżnicowaną pulę genetyczną można znaleźć u dzikich krewniaków roślin uprawnych, które – ze względu na duże możliwości adaptacyjne do szerokiego zakresu siedlisk i warunków środowiskowych – stanowią cenny rezerwuar genów istotnych z punktu widzenia hodowli roślin (Brozynska i in. 2016; Dempewolf i in. 2017; Prohens i in. 2017). W tym kontekście dzikie gatunki rodzaju *Daucus* mogłyby odegrać ważną rolę w procesie doskonalenia współczesnego rolnictwa, będąc potencjalnym źródłem takich genów – przydatnych w procesie otrzymywania nowych odmian roślin uprawnych, np. odpornych na choroby i stresy abiotyczne, wyżej plonujących, męskosterylnych lub o lepszej wartości odżywczej (Grzebelus i in. 2011; Arbizu i in. 2014).

Rodzaj *Daucus* stanowi doskonały model do badań nad ewolucją kariotypów. Obejmuje marchew uprawną – podgatunek o zsekwencjonowanym genomie – oraz około 40 dzikich gatunków, które różnią się pod względem wielkości genomu i liczby chromosomów. Jednakże w przypadku *Daucus* podstawowe dane cytogenetyczne są wciąż niepełne, a dla niektórych gatunków literatura podaje sprzeczne informacje na ten temat. Rozbieżność ta może wynikać z błędnej identyfikacji gatunków, co jest związane z problematyczną taksonomią tego rodzaju (Iovene i Grzebelus 2019).

Wielkość genomu – zdefiniowana jako ilość DNA w haploidalnym genomie organizmu (Greilhuber i in. 2005) i wyrażana w jednostkach wagowych (pikogramach) lub liczbie par zasad (1 pg = 978 Mpz; Doležel i in. 2003) – jest cechą gatunkową, która może dostarczyć cennych informacji o historii ewolucyjnej gatunków. Wiedza o wielkości genomu jest przydatna w badaniach z zakresu systematyki, filogenetyki i fitogeografii, w wyjaśnianiu powiązań taksonomicznych między gatunkami, pozwala ujawnić błędne klasyfikacje w kolekcjach zasobów genowych przechowywanych w bankach genów, a także pomaga zidentyfikować poliploidie/aneuploidie czy duże rearanżacje strukturalne. Znajomość tego parametru jest również niezbędna w projektach sekwencjonowania genomów, ponieważ zależą od niego ich skala i koszty (Bennett i Leitch 2011; Leitch i Leitch 2013; Nowicka i in. 2016b; Yan i in. 2016; Sliwinska 2018; Melichárková i in. 2019; Redpath i in. 2022; Ghanbari i in. 2023; Wang i in. 2023).

Ponadto – mimo że żyjemy obecnie w erze genomiki porównawczej, w której coraz więcej genów i genomów jest sekwencjonowanych, dostarczając cennych informacji o ewolucji organizmów – wciąż warto gromadzić dane morfologiczne, ponieważ mogą one służyć jako wsparcie lub uzupełnienie dla wniosków sformułowanych na podstawie danych molekularnych (Liao i in. 2013; Xiao i in. 2021; El-Taher i in. 2023). W przypadku Apiaceae doskonałym źródłem takich danych są pyłek i owoce, które okazały się niezwykle przydatne w badaniach taksonomicznych i ewolucyjnych tej rodziny (Spalik i in. 2001; Liu i in. 2003, 2006, 2009; Kljuykov i in. 2004; Khajepiri i in. 2010; Güner i in. 2011; Akalın i in. 2016; Liu i Downie 2017; Wojewódzka i in. 2019; Baczyński i in. 2021; Baser i in. 2021; Birjees i in. 2022; Özkök i in. 2022). Pomimo że wiele gatunków należących do różnych rodzajów rodziny Apiaceae zostało poddanych analizom palinologicznym (PalDat 2023), w literaturze wciąż brakuje takich danych dla dzikich przedstawicieli *Daucus*. Z kolei w przypadku badań karpologicznych dostępne dane dla tego rodzaju (Sáenz Lain 1981; Mezghani i in. 2014) są również niewystarczające.

Dlatego też w świetle uaktualnionej klasyfikacji rodzaju *Daucus* wciąż istnieje potrzeba weryfikacji liczby chromosomów i wielkości genomu u jego dzikich przedstawicieli. Ponadto dalsze badania z zakresu cytogenetyki molekularnej, zawartości jądrowego DNA i sekwencji powtarzalnych w obrębie *Daucus* mogą pomóc w zrozumieniu mechanizmów biorących udział w specjacji tych gatunków i organizacji ich genomu na poziomie chromosomowym. Stąd też badania (cytogenetyczne, morfologiczne i anatomiczne) ukierunkowane na bliższe poznanie rodzaju *Daucus* i lepsze zrozumienie powiązań między jego gatunkami mogą w szerszej perspektywie przyczynić się do rozwoju przyszłych programów hodowlanych marchwi.

4. Hipotezy i cele badawcze

Ostatnie prace nad poznaniem genomu marchwi uprawnej (*Daucus carota* subsp. *sativus*), a także rosnąca świadomość znaczenia dzikich krewniaków roślin uprawnych dla kształtowania współczesnego rolnictwa stały się asumptem do podjęcia badań nad dzikimi gatunkami rodzaju *Daucus*. W niniejszej pracy doktorskiej sformułowano następujące hipotezy badawcze:

1. Zidentyfikowane w genomie marchwi uprawnej sekwencje powtarzalne CentDc występują również w genomach jej dzikich krewniaków i są zlokalizowane w przewężeniach pierwotnych (centromerach) ich chromosomów, dzięki czemu mogą służyć jako centromerowo specyficzne markery cytogenetyczne.

2. Gatunki rodzaju *Daucus* różnią się między sobą pod względem zawartości jądrowego DNA, morfologii pyłku oraz morfologii i anatomii owoców.

3. Zawartość jądrowego DNA, cechy palinologiczne oraz cechy morfo-anatomiczne owoców mogą pomóc w identyfikacji gatunków rodzaju *Daucus*.

Aby zweryfikować tak postawione hipotezy, wyznaczono następujące cele badawcze:

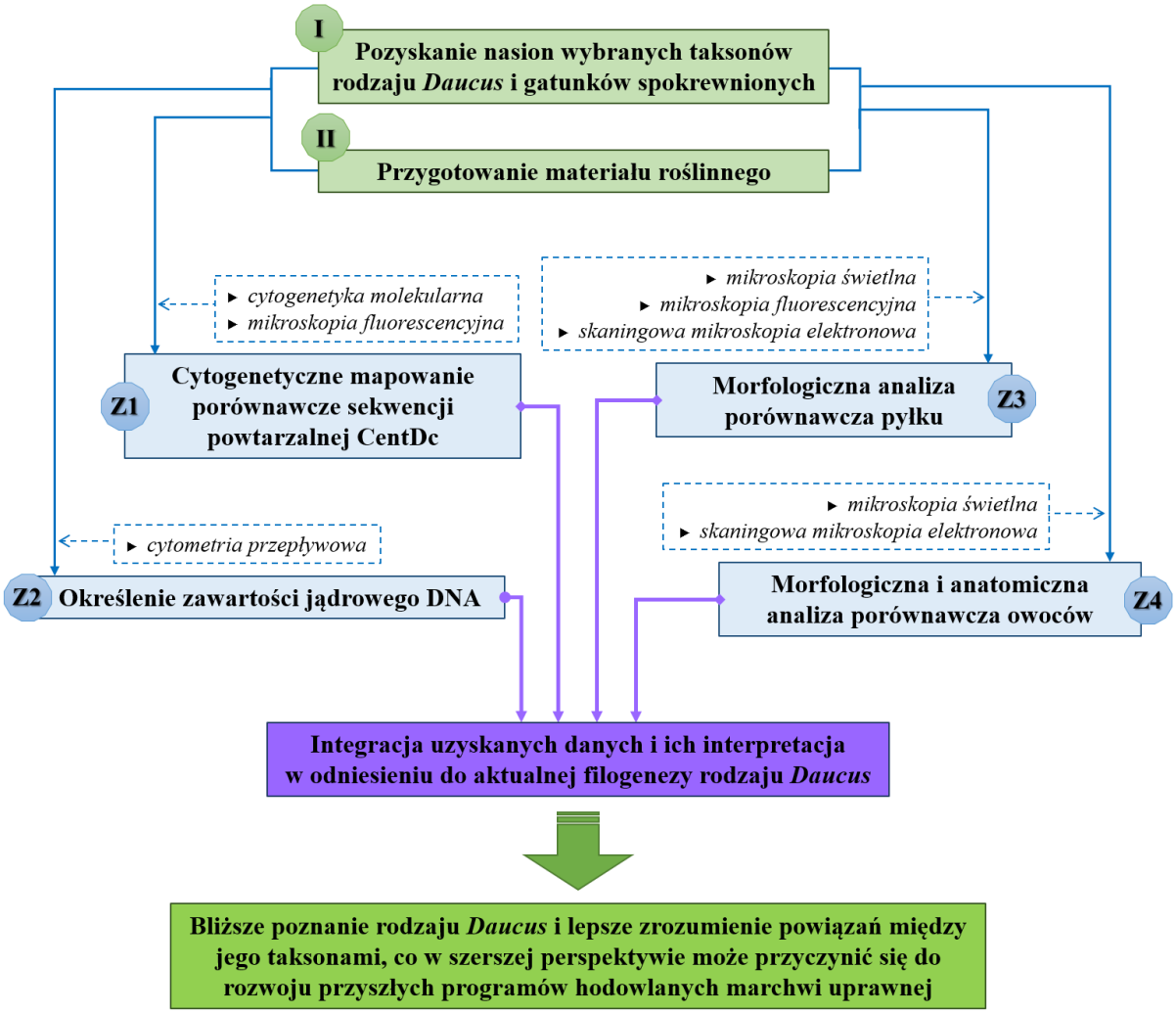
1. Porównawcze mapowanie cytogenetyczne sekwencji powtarzalnej CentDc (zidentyfikowanej w genomie marchwi uprawnej) u wybranych taksonów (gatunków i podgatunków) rodzaju *Daucus* i gatunków spokrewnionych (**Publikacja 1**).

2. Określenie zawartości jądrowego DNA w genomach wybranych taksonów rodzaju *Daucus* i gatunków spokrewnionych (**Publikacja 2**).

3. Analiza porównawcza morfologii pyłku oraz morfologii i anatomii owoców u wybranych taksonów rodzaju *Daucus* i gatunków spokrewnionych (**Publikacje 2** i **3**).

5. Materiały i metody

Realizowany w ramach rozprawy doktorskiej plan badawczy (**Rycina 2**) składał się z czterech niezależnych zadań badawczych (**Z1–4**) poprzedzonych dwoma zadaniami wstępnymi (**I**, **II**) o charakterze przygotowawczym, obejmującymi (**I**) pozyskanie nasion (owoców) wybranych taksonów z banków genów i (**II**) przygotowanie materiału roślinnego, tj. uprawa roślin oraz zebranie i zabezpieczenie materiału badawczego (korzenie, liście, pyłek, owoce) do dalszych analiz.



Rycina 2. Schemat planu badawczego. (I, II) – zadania wstępne; (Z1–4) – zadania badawcze; ramki (linia przerywana) wskazują narzędzia badawcze zastosowane w ramach realizacji danego zadania badawczego

Materiał roślinny stanowiły wybrane obiekty (ang. *accessions*) taksonów rodzaju *Daucus* – w tym dzikie gatunki, podgatunki *D. carota* oraz linie/odmiany marchwi uprawnej – oraz gatunków spokrewnionych należących do rodzajów *Ammi*, *Astrodaucus*, *Caucalis*, *Orlaya* i *Torilis* (**Tabela 1**).

Szczegółowy opis metod stosowanych w trakcie realizacji wyżej wymienionych zadań badawczych został zawarty w sekcjach „Materials and Methods" poszczególnych publikacji wchodzących w skład rozprawy doktorskiej.

Tabela 1. Wykaz taksonów rodzaju *Daucus* i gatunków spokrewnionych wykorzystywanych w ramach realizacji pracy doktorskiej

Takson[a]	2*n*[b]	Pochodzenie nasion[c] / Numer obiektu	Kraj pochodzenia	Publikacja[d]		
				P1	P2	P3
Klad *Daucus* I						
D. aureus Desf.	22	USDA / PI 295854	Izrael	×		
D. aureus	22	USDA / PI 319403	Izrael	×	×	×
D. carota subsp. *capillifolius* (Gilli) C. Arbizu	18	USDA / PI 279764	Libia	×	×	×
D. carota subsp. *capillifolius*	18	USDA / Ames 30198	Tunezja	×		
D. carota subsp. *carota* L.	18	USDA / PI 274297	Pakistan	×		
D. carota subsp. *carota*	18	USDA / PI 478369	Chiny	×		
D. carota subsp. *carota*	18	USDA / PI 478861	Francja	×		
D. carota subsp. *carota*	18	USDA / PI 652393	Turcja	×		
D. carota subsp. *gummifer* (Syme) Hook. f.	18	USDA / PI 478883	Francja	×		
D. carota subsp. *gummifer*	18	USDA / Ames 26383	Portugalia	×		
D. carota subsp. *maximus* (Desf.) Ball	18	USDA / Ames 26408	Portugalia	×		
D. carota subsp. *sativus* Hoffm.	18	RZ / DH1*	Holandia	×	×	×
D. carota subsp. *sativus*	18	'Dolanka'**	Polska	×	×	×
D. carota subsp. *sativus*	18	'Amsterdam'**	Polska	×		
D. crinitus Desf.	22	USDA / PI 652414	Portugalia	×		
D. muricatus (L.) L.	22	USDA / PI 295863	Hiszpania	×	×	×
D. muricatus	22	USDA / Ames 29090	Tunezja	×		
D. pumilus (L.) Hoffmanns. & Link	16	USDA / PI 662301	Tunezja	×		
D. rouyi Spalik & Reduron	20	USDA / PI 674284	Tunezja	×	×	×
D. sahariensis Murb.	18	USDA / Ames 29096	Tunezja	×	×	×
D. sahariensis	18	USDA / Ames 29097	Tunezja	×		
D. syrticus Murb.	18	USDA / Ames 29108	Tunezja	×	×	×
Klad *Daucus* II						
D. conchitae Greuter	22	USDA / Ames 25835	Turcja	×	×	×
D. glochidiatus (Labill.) Fisch & C.A. Mey	44	USDA / PI 285038	Australia	×	×	×
D. guttatus Sm.	20	USDA / PI 652233	Iran		×	×
D. involucratus Sm.	22	USDA / PI 652332	Grecja	×	×	×
D. involucratus	22	USDA / PI 652355	Turcja	×		
D. littoralis Sm.	20	USDA / PI 295857	Izrael	×	×	×
D. pusillus Michx.	22	USDA / PI 349267	Urugwaj	×	×	×
Gatunki spokrewnione						
Ammi visnaga (L.) Lam.	20	IPK / AMMI 25	Niemcy	×		
Astrodaucus littoralis (M. Bieb.) Drude	20	USDA / PI 277064	Azerbejdżan	×		
Caucalis platycarpos L.	20	USDA / PI 649446	Niemcy	×	×	×
Orlaya daucoides (L.) Greuter	16	USDA / PI 649477	Turcja	×	×	×
O. daucorlaya Murb.	14	USDA / PI 649478	Grecja		×	×
Torilis arvensis (Huds.) Link	12	USDA / PI 649391	Syria	×	×	×
T. arvensis	12	USDA / PI 649394	Turcja	×		

[a] Klasyfikacja taksonomiczna według Arbizu i in. (2014) i Banasiaka i in. (2016)

[b] Somatyczna liczba chromosomów (Iovene i in. 2008; Rice i in. 2015)

[c] IPK – Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Niemcy; RZ – firma hodowlana Rijk Zwaan, Lier, Holandia; USDA – USDA-ARS North Central Regional Plant Introduction Station (NCRPIS), Ames, Iowa, USA; * DH1 – linia podwojonego haploida marchwi typu nantejskiego o pomarańczowej barwie korzenia; ** odmiany uprawne marchwi, nasiona pochodziły ze źródeł komercyjnych

[d] P1 – Kadluczka i Grzebelus (2021); P2 – Kadluczka i in. (2022); P3 – Kadluczka i Grzebelus (2022)

6. Streszczenia załączonych publikacji – najważniejsze wyniki

6.1. Publikacja nr 1

Kadluczka D.✉, Grzebelus E.✉ 2021. Using carrot centromeric repeats to study karyotype relationships in the genus *Daucus* (Apiaceae). *BMC Genomics*, 22, 508.
DOI: https://doi.org/10.1186/s12864-021-07853-2

Punktacja MEiN$_{2021}$: **140**
IF$_{2021}$: **4,558**

Celem badań zawartych w tej publikacji było określenie lokalizacji chromosomowej sekwencji powtarzalnej CentDc – zidentyfikowanej w genomie marchwi uprawnej – u wybranych taksonów rodzaju *Daucus* i gatunków spokrewnionych za pomocą fluorescencyjnej hybrydyzacji *in situ* (FISH). Celem pobocznym tej pracy było przeprowadzenie dokładnych analiz kariomorfologicznych u części z tych taksonów.

Materiał roślinny stanowiły 34 obiekty (ang. *accessions*) reprezentujące 22 taksony (gatunki i podgatunki), w tym 28 obiektów rodzaju *Daucus* oraz 6 obiektów gatunków spokrewnionych należących do rodzajów *Ammi*, *Astrodaucus*, *Caucalis*, *Orlaya* i *Torilis*. Wśród obiektów rodzaju *Daucus* było 16 obiektów dzikich gatunków oraz 12 obiektów wybranych podgatunków *D. carota*, w tym również trzy obiekty marchwi uprawnej (jedna linia hodowlana i dwie odmiany uprawne).

Do przeprowadzenia porównawczej analizy FISH wykorzystano syntetyczną, bezpośrednio znakowaną sondę o długości 36 nukleotydów, którą stosowano we wcześniejszych badaniach nad kariotypem marchwi (Nowicka i in. 2012, 2016). Sonda ta została zaprojektowana na podstawie sekwencji konsensusowej dla podjednostek oryginalnego powtórzenia centromerowego CentDc marchwi (Cavagnaro i in. 2009; Iovene i in. 2011).

Porównawcza analiza FISH wykazała obecność sekwencji CentDc w genomach 26 obiektów (reprezentujących 15 taksonów) rodzaju *Daucus* i jednego gatunku spokrewnionego (*Astrodaucus littoralis*). U pozostałych siedmiu obiektów nie zaobserwowano sygnałów fluorescencyjnych, co może świadczyć o braku lub niskiej liczbie kopii tych powtórzeń w ich genomach.

W przypadku 20 obiektów, które reprezentowały 11 taksonów rodzaju *Daucus* (sześć dzikich gatunków i pięć podgatunków *D. carota*), sonda CentDc hybrydyzowała do obszarów centromerowych wszystkich chromosomów tych obiektów, przy czym u gatunku *D. aureus*

jedna para chromosomów zawierała – poza sygnałami centromerowymi – dodatkowe sygnały. W obrębie każdego z tych obiektów intensywność sygnałów fluorescencyjnych pomiędzy poszczególnymi chromosomami różniła się, co sugeruje różną liczbę kopii tych powtórzeń.

Pozostałe FISH-pozytywne gatunki wykazywały wzór hybrydyzacyjny zróżnicowany pod względem liczby par chromosomów zawierających powtórzenia CentDc. U gatunku *D. muricatus* (klad *Daucus* I) zaobserwowano sygnały zlokalizowane w regionach centromerowych i przycentromerowych ośmiu par chromosomów. Z kolei u *D. glochidiatus* (klad *Daucus* II) wykryto pięć par chromosomów z centromerowo specyficznymi sygnałami. Najmniej sygnałów zaobserwowano u gatunków *D. involucratus* i *D. conchitae* (oba należące do kladu *Daucus* II), u których sygnały znajdowały się w obszarach centromerowych odpowiednio dwóch i jednej pary chromosomów.

W przypadku *Astrodaucus littoralis* – jedynego gatunku nienależącego do rodzaju *Daucus*, u którego wykryto obecność powtórzeń CentDc – sygnały znajdowały się na czterech parach chromosomów, jednakże nie udało się jednoznacznie określić, czy sygnały te obejmowały regiony centromerowe.

Dzięki wykazaniu, że sekwencja CentDc może służyć jako marker cytogenetyczny do identyfikacji centromerów w rodzaju *Daucus* oraz wskazaniu taksonów, u których sygnały CentDc występowały w regionach centromerowych wszystkich chromosomów, u wybranych obiektów możliwe było dokonanie precyzyjnych pomiarów chromosomów.

6.2. Publikacja nr 2

Kadluczka D.✉, Sliwinska E., Grzebelus E.✉ 2022. Combining genome size and pollen morphology data to study species relationships in the genus *Daucus* (Apiaceae). *BMC Plant Biology*, 22, 382.

DOI: https://doi.org/10.1186/s12870-022-03743-1

Punktacja $MEiN_{2021}$: **140**

IF_{2021}: **5,260**

Głównym celem tej pracy było oszacowanie zawartości jądrowego DNA w genomach wybranych taksonów rodzaju *Daucus* i gatunków spokrewnionych za pomocą cytometrii przepływowej oraz określenie ich morfologii pyłku przy użyciu mikroskopii świetlnej i skaningowej mikroskopii elektronowej (SEM).

Materiał roślinny stanowiło 17 taksonów (gatunki i podgatunki), w tym 12 dzikich taksonów *Daucus*, dwa obiekty marchwi uprawnej i cztery gatunki spokrewnione należące do rodzajów *Caucalis*, *Orlaya* i *Torilis*.

Analiza cytometryczna wykazała 3,2-krotne zróżnicowanie zawartości jądrowego DNA wśród taksonów rodzaju *Daucus*, która mieściła się w zakresie od 0,999 pg u marchwi uprawnej ($2n$ = 18) do 3,228 pg u *D. littoralis* ($2n$ = 20). Z kolei u gatunków spokrewnionych wartość 2C DNA wahała się od 1,775 pg u *C. platycarpos* ($2n$ = 20) do 2,882 pg u *T. arvensis* ($2n$ = 12). Taksony należące do kladu *Daucus* I cechowała mniejsza wielkość genomu (1C DNA) w porównaniu z przedstawicielami kladu *Daucus* II, z wyjątkiem *D. muricatus* ($2n$ = 22; *Daucus* I), którego genom był dwukrotnie większy niż genom pozostałych taksonów tego kladu. Ponadto nie zaobserwowano zależności między zawartością jądrowego DNA a liczbą chromosomów w rodzaju *Daucus*.

Analiza palinologiczna wykazała, że pyłek badanych taksonów różni się pod względem wielkości i kształtu. W przypadku *Daucus* średnia długość osi biegunowej ziaren pyłku mieściła się w zakresie od 21,19 µm u *D. glochidiatus* do 40,38 µm u *D. aureus*. Z kolei spośród gatunków spokrewnionych najmniejsze ziarna pyłku zaobserwowano u *T. arvensis* (26,01 µm), natomiast największe – u *O. daucorlaya* (49,86 µm). Pod względem kształtu – na podstawie stosunku długości osi biegunowej (P) ziaren pyłku do długości ich osi równikowej (E) – pyłek badanych taksonów sklasyfikowano jako mniej (P/E = 1,33–2,00; ang. *prolate*) lub bardziej (P/E > 2,00; ang. *perprolate*) wydłużony, przy czym ten drugi typ był charakterystyczny tylko dla dwóch gatunków *Daucus* (*D. aureus* i *D. rouyi*) oraz dla gatunków spokrewnionych. Ponadto zaobserwowano silną pozytywną korelację pomiędzy parametrami P i E (r = 0,834; $p < 0,001$) oraz pomiędzy P i P/E (r = 0,823; $p < 0,001$).

Strukturę sporodermy ziaren pyłku scharakteryzowano za pomocą SEM. Wszystkie ziarna pyłku cechowała obecność trzech porusów (apertur), natomiast pod względem urzeźbienia egzyny wyróżniono kilka typów morfologicznych: prążkowany (ang. *striate*) u *D. conchitae* i *D. rouyi*; pomarszczony (ang. *rugulate*) u *D. involucratus* i *D. carota* subsp. *capillifolius*; perforowany u *D. guttatus*, *O. daucoides* i *O. daucorlaya*; u pozostałych taksonów zaobserwowano mieszane typy urzeźbienia egzyny.

6.3. Publikacja nr 3

Kadluczka D., Grzebelus E.✉ 2022. Comparative fruit morphology and anatomy of wild relatives of carrot (*Daucus*, Apiaceae). *Agriculture*, 12, 2104.
DOI: https://doi.org/10.3390/agriculture12122104

Punktacja MEiN$_{2021}$: **100**

IF$_{2021}$: **3,408**

Celem tej pracy było zbadanie cech morfologicznych i anatomicznych owoców wybranych taksonów rodzaju *Daucus* i gatunków spokrewnionych przy użyciu mikroskopii świetlnej i skaningowej mikroskopii elektronowej (SEM) oraz ocena ich wartości diagnostycznej.

Materiał badawczy stanowiły owoce 17 taksonów, w tym 12 dzikich taksonów *Daucus*, dwóch obiektów marchwi uprawnej i czterech gatunków spokrewnionych należących do rodzajów *Caucalis*, *Orlaya* i *Torilis* – te same obiekty, które wykorzystano w Publikacji nr 2.

Owocami badanych taksonów były rozłupnie składające się z dwóch jednonasiennych rozłupek o kształcie od jajowatego do podłużnego. Owoce tych taksonów były zaopatrzone w kolce – z wyjątkiem owoców *D. rouyi*, które były uskrzydlone. Każda rozłupka zawierała pięć żeber pierwszorzędowych i cztery żebra drugorzędowe (dwa grzbietowe i dwa boczne), przy czym u większości taksonów te pierwsze były mniej lub bardziej niepozorne, pokryte włoskami lub cierniami, natomiast te drugie – większe i wyraźnie widoczne; jedynie u *T. arvensis* zaobserwowano na powierzchni owoców liczne rzędy dodatkowych żeber drugorzędowych.

Średnia długość owoców u *Daucus* mieściła się w zakresie od 2,1 mm u marchwi uprawnej do 8,4 mm u *D. rouyi*, podczas gdy ich szerokość zawierała się w przedziale od 1,1 mm u *D. conchitae* i *D. involucratus* do 7,7 mm u *D. rouyi*. Natomiast u gatunków spokrewnionych długość i szerokość owoców wahały się w zakresach odpowiednio 2,7–11,4 i 2,8–5,8 mm.

U zdecydowanej większości taksonów *Daucus* urzeźbienie powierzchni owoców – jak uwidoczniono za pomocą SEM – było mniej lub bardziej pomarszczone; owoce pozostałych cechowały się mieszanym wzorem urzeźbienia. Najłatwiej odróżnialnym taksonem okazał się *D. aureus*, którego powierzchnia owoców była gęsto pokryta guzkami. Z kolei u gatunków spokrewnionych zaobserwowano owoce o powierzchni gładkiej (*C. platycarpos*), pofałdowanej (*O. daucoides* i *O. daucorlaya*) oraz guzowatej (*T. arvensis*).

W przekroju poprzecznym rozłupki prawie wszystkich badanych taksonów były lekko spłaszczone grzbietowo, jedynie u *C. platycarpos* były one lekko spłaszczone bocznie.

U wszystkich taksonów owocnia (perykarp) składała się z trzech typowych warstw: zewnętrznego egzokarpu, środkowego mezokarpu i wewnętrznego endokarpu, a jej grubość mieściła się w zakresie od 28 do 132 µm. Jedynie u gatunków z rodzaju *Orlaya* stwierdzono obecność dodatkowej struktury – tak zwanego hypendokarpu, czyli wewnętrznej części mezokarpu, w której występują warstwy zdrewniałych włókien. Ponadto w owocach *D. glochidiatus* i *D. sahariensis* zaobserwowano, że egzokarp pokrywający ich żebra drugorzędowe zbudowany jest z komórek zawierających charakterystyczne trójkątne wypustki.

Owoce większości taksonów *Daucus* cechowały się obecnością dobrze rozwiniętych kanałów wydzielniczych (ang. *vittae*), ich szerokość wahała się od 33 do 168 µm; wyjątek stanowił *D. aureus*, którego owoce były pozbawione tych struktur. Każda rozłupka zawierała sześć kanałów wydzielniczych – cztery znajdujące się w żebrach drugorzędowych (ang. *vallecular vittae*) oraz dwa od strony brzusznej (ang. *commissural vittae*). W przypadku gatunków spokrewnionych liczba i rozmieszczenie tych kanałów były takie same jak u *Daucus*, przy czym największe zaobserwowano u *O. daucorlaya* (329 µm).

W mezokarpie pod każdym żebrem pierwszorzędowym przebiegały wiązki przewodzące, przy czym u *D. aureus* wiązki znajdujące się od strony brzusznej były ze sobą połączone, układając się w charakterystyczny kształt przypominający literę M.

7. Podsumowanie i wnioski

W ramach prezentowanej rozprawy doktorskiej przeprowadzono szereg badań ukierunkowanych na bliższe poznanie rodzaju *Daucus*. Wyniki tych badań poszerzają aktualny stan wiedzy o dzikich krewniakach marchwi uprawnej, dostarczając nowych danych z zakresu cytogenetyki, morfologii pyłku oraz morfologii i anatomii owoców. W efekcie tych prac zaobserwowano szeroki zakres zmienności analizowanych cech, co dowodzi, że rodzaj *Daucus* stanowi doskonały model do badań porównawczych.

Uzyskane dane mogą być przydatne z punktu widzenia wyjaśniania powiązań taksonomicznych między gatunkami tego rodzaju, a także mogą pomóc w prawidłowej identyfikacji zasobów genowych zgromadzonych w bankach genów. W szerszej perspektywie otrzymane wyniki mogą mieć istotne znaczenie dla interpretacji trendów ewolucyjnych w rodzaju *Daucus* oraz przyczynić się do rozwoju przyszłych programów hodowlanych marchwi. Jednak aby lepiej zrozumieć powiązania między przedstawicielami rodzaju *Daucus* (w kontekście filogenetycznym), potrzebne są dalsze badania obejmujące pozostałe taksony rodzaju – w tym te niedawno do niego włączone.

Przeprowadzone badania pozwoliły na sformułowanie następujących wniosków:

1. Zidentyfikowane w genomie marchwi uprawnej powtórzenia CentDc występują również w genomach innych przedstawicieli rodzaju *Daucus*, a także w genomie spokrewnionego gatunku *Astrodaucus littoralis*.

2. Obecność powtórzeń CentDc w genomach dzikich krewniaków marchwi sugeruje, że sekwencja ta występowała w genomie ich wspólnego przodka. Z kolei w przypadku gatunków, u których ich nie wykryto, na drodze ewolucji mogło dojść do utraty tych powtórzeń lub ich zastąpienia przez inne sekwencje satelitarne; możliwe jest również, że liczba kopii powtórzeń CentDc była zbyt niska, aby można je było wykryć za pomocą narzędzi cytogenetyki molekularnej.

3. Powtórzenia CentDc mogą służyć jako markery cytogenetyczne pozwalające na identyfikację centromerów w chromosomach taksonów, u których je wykryto.

4. Gatunki rodzaju *Daucus* różnią się między sobą pod względem zawartości jądrowego DNA.

5. Znaczne różnice w wielkości genomu zaobserwowane u dzikich gatunków rodzaju *Daucus* sugerują, że w trakcie specjacji w ich genomach wystąpiły duże rearanżacje chromosomalne i/lub nagromadzenie powtarzalnych sekwencji DNA.

6. Gatunki rodzaju *Daucus* różnią się między sobą pod względem cech palinologicznych oraz cech morfologicznych i anatomicznych owoców.

7. Zawartość jądrowego DNA, cechy morfologiczne ziaren pyłku, a także cechy morfologiczne i anatomiczne owoców – zwłaszcza w połączeniu – mogą pomóc w identyfikacji niektórych gatunków rodzaju *Daucus*.

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Using carrot centromeric repeats to study karyotype relationships in the genus *Daucus* (Apiaceae)

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Abstract

Background: In the course of evolution, chromosomes undergo evolutionary changes; thus, karyotypes may differ considerably among groups of organisms, even within closely related taxa. The genus *Daucus* seems to be a promising model for exploring the dynamics of karyotype evolution. It comprises some 40 wild species and the cultivated carrot, a crop of great economic significance. However, *Daucus* species are very diverse morphologically and genetically, and despite extensive research, the taxonomic and phylogenetic relationships between them have still not been fully resolved. Although several molecular cytogenetic studies have been conducted to investigate the chromosomal structure and karyotype evolution of carrot and other *Daucus* species, detailed karyomorphological research has been limited to carrot and only a few wild species. Therefore, to better understand the karyotype relationships within *Daucus*, we (1) explored the chromosomal distribution of carrot centromeric repeats (CentDc) in 34 accessions of *Daucus* and related species by means of fluorescence in situ hybridization (FISH) and (2) performed detailed karyomorphological analysis in 16 of them.

Results: We determined the genomic organization of CentDc in 26 accessions of *Daucus* (belonging to both *Daucus* I and II subclades) and one accession of closely related species. The CentDc repeats were present in the centromeric regions of all chromosomes of 20 accessions (representing 11 taxa). In the other *Daucus* taxa, the number of chromosome pairs with CentDc signals varied depending on the species, yet their centromeric localization was conserved. In addition, precise chromosome measurements performed in 16 accessions showed the inter- and intraspecific karyological relationships among them.

Conclusions: The presence of the CentDc repeats in the genomes of taxa belonging to both *Daucus* subclades and one outgroup species indicated the ancestral status of the repeat. The results of our study provide useful information for further evolutionary, cytotaxonomic, and phylogenetic research on the genus *Daucus* and may contribute to a better understanding of the dynamic evolution of centromeric satellites in plants.

Keywords: Crop wild relatives, Cytotaxonomy, Karyomorphology, Molecular cytogenetics, Plant chromosomes

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Background

Chromosomes undergo evolutionary changes in the course of evolution; thus, karyotypes may differ considerably among groups of organisms, even within closely related taxa. Hence, the study of chromosomes by means of karyotypic features, including the number, size, centromere position, number and position of secondary constrictions, symmetry, and banding patterns of the chromosome complement, has been widely used in taxonomy (cytotaxonomy), systematics, and phylogeny, thus greatly contributing to our understanding of evolutionary processes. It has also been confirmed that repetitive DNA sequences are tightly associated with chromosome evolution in plants [1–3].

A substantial portion of plant genomes are composed of various types of repetitive DNA sequences, classified as tandem or dispersed, according to the genomic organization of their repeat units. The dispersed repeats are scattered throughout the genome (transposable elements), whereas tandem repeats appear in the form of large arrays consisting of thousands or millions of monomers, comprising microsatellites, minisatellites, and satellite DNA [4, 5]. Unlike low-copy-number sequences, repetitive elements are highly variable and evolve more rapidly, leading to changes in the abundance and chromosomal distribution of their copies. Furthermore, due to their high copy number and tendency to cluster, they are excellent probes for fluorescence in situ hybridization (FISH), a powerful molecular cytogenetic technique, providing valuable information on their physical localization, thus making them advantageous for comparative studies concerning evolutionary relationships between species [6, 7]. Of these, satellite DNA has greatly contributed to our knowledge on chromosome and genome evolution, as well as the phylogeny of species. This class of repeats is preferentially associated with specific chromosomal segments, most frequently found at centromeric, pericentromeric, and subtelomeric regions but also at intercalary positions [4, 5, 8]. Chromosomal sites rich in satellite DNA usually exhibit a unique banding pattern, which makes them ideal as cytogenetic markers for the identification of individual chromosomes; therefore they are useful for karyotype descriptions [9–14]. FISH with satellite DNA-based probes has also been successfully applied for the understanding of chromosomal evolution in several agronomically important plant species, including sugar beet [8], maize [15], radish [16], common bean [17], spinach [18], and quinoa [19].

Carrot (*Daucus carota* subsp. *sativus* Hoffm.), belonging to the large and complex family Apiaceae, is the most significant member of the genus *Daucus*, being a major source of vitamin A precursors (α- and β-carotene) in the human diet [20]. The genus comprises some 40 wild species known to be morphologically and genetically diverse. For this reason, despite numerous research efforts at various levels (morphological, anatomical, and molecular), the taxonomic delimitation and phylogenetic relationships between them have still not been fully resolved [21–28]. The correlation of the *Daucus* taxonomy with its phylogeny is a challenging task because the clades inferred from molecular data have no obvious morphological synapomorphies allowing the recognition of their taxa. On the basis of recent molecular studies using plastid and nuclear DNA sequences *Daucus* species were divided into two subclades: *Daucus* I and *Daucus* II, of which *Daucus* I groups the wild ancestor of the cultivated carrot and its subspecies, several Mediterranean *Daucus* species, and some members of other genera (*Athamanta*, *Pachyctenium*, *Pseudorlaya*, *Tornabenea*), whereas *Daucus* II includes the remaining *Daucus* members, along with its American and Australian representatives [23, 25]. A recent reevaluation of *Daucus* by Banasiak et al. [26], in which the nuclear ribosomal DNA ITS and three chloroplast markers were used, has expanded the genus to include the following taxa: *Agrocharis* Hochst. (four species), *Melanoselinum* Hoffm. (one species), *Monizia* Lowe (one species), *Pachyctenium* Maire & Pamp. (one species), *Pseudorlaya* (Murb.) Murb. (two species), *Rouya* Coincy (one species), *Tornabenea* Parl. (six species), *Athamanta dellacellae* E.A. Durand & Barratte, and *Cryptotaenia elegans* Webb ex Bolle.

The great diversity of *Daucus* species makes it a promising model for cytotaxonomic and evolutionary research. To date, several molecular cytogenetic studies have been conducted to investigate the chromosomal structure and karyotype evolution of carrot and other *Daucus* [29–34]. Nonetheless, detailed karyomorphological studies in *Daucus* have been limited to carrot [29, 32, 35–38] and only a few wild species [29].

When dealing with chromosomes of different *Daucus* species, it is often difficult to obtain metaphase spreads suitable for precise measurements. This is due to the relative morphological uniformity of the chromosomes, in which the position of the primary constrictions is not always possible to unequivocally determine. In carrot, however, this obstacle has recently been overcome through the identification of a carrot centromeric repeat, named CentDc, which is typically composed of four 39–40-bp monomers that vary slightly in sequence [30, 39]. Consequently, a consensus sequence derived from these monomers was used as a FISH probe, along with some other repetitive probes, for hybridization to metaphase chromosomes of carrot, enabling detailed karyotype measurements and differentiation of its individual chromosomes [32]. In addition to *Daucus carota*, comparative in silico analysis was conducted on five other

Daucus species, indicating the presence of CentDc-like sequences in three of them, whereas the two remaining ones were further analyzed by FISH to confirm the absence of these repeats [33]. These findings suggest the hypothesis that carrot centromeric repeats are widespread in the genus *Daucus* and that their chromosomal distribution can be examined by molecular cytogenetics. However, the detailed and comprehensive comparative FISH mapping of carrot centromeric sequences in *Daucus* has not been reported before.

In this study, we aimed to address how carrot centromeric repeats have evolved; therefore, we employed a FISH-based approach to explore the chromosomal distribution of these repeats in 34 accessions of *Daucus* and related taxa. Subsequently, we identified taxa that – like carrot – carry CentDc repeats in the primary constrictions of all chromosomes, which, in turn, enabled us to take precise karyotype measurements. Moreover, these data allowed us to discuss the relationships among *Daucus* species based on their karyotype features.

Results

Comparative FISH mapping of CentDc repeats

For comparative FISH analysis, we selected 34 accessions of *Daucus* (belonging to both *Daucus* I and II subclades) and related species (Table 1).

FISH on metaphase chromosome spreads with CentDc (hereinafter referred to as a 36-nucleotide sequence based on the consensus sequence corresponding to a subrepeat of the original CentDc repeat; see 'Methods') were used as a probe and displayed a clear hybridization pattern in 27 out of 34 accessions examined in this study (Fig. 1 and 2a–h). Seven other accessions, representing six taxa, did not show any fluorescence signals, suggesting either the absence or low copy number of CentDc repeats in their genomes. Metaphase chromosomes of these FISH-negative accessions are shown in Fig. 2i–o.

In the case of 20 accessions, representing 11 taxa (10 taxa belonging to the *Daucus* I subclade, 1 taxon belonging to the *Daucus* II subclade), the CentDc probe hybridized to the centromeric regions of all chromosomes (Fig. 1). In each accession, the fluorescence intensity of the FISH signals varied between different chromosomes, indicating differences in copy number of CentDc repeats. However, these differences in the fluorescence intensity were not sufficient to enable the identification of all homologous chromosomes. Among these taxa, both accessions of *D. aureus* ($2n = 22$) had one chromosome pair showing additional signals of CentDc – along with the centromeric ones – observed in the interstitial regions of the long arms (Fig. 1a–b, *arrows*).

The other FISH-positive taxa displayed different hybridization patterns that varied in terms of the number of chromosome pairs with CentDc signals. Among them, both accessions of *D. muricatus* ($2n = 22$) (*Daucus* I subclade) had eight CentDc-carrying chromosome pairs in which the signals were located either in the centromeric or pericentromeric regions (Fig. 2a–b). For *D. glochidiatus* ($2n = 44$) (*Daucus* II subclade), the only polyploid species analyzed here, centromeric signals were revealed on five chromosome pairs, of which one pair was marked by distinctly strong signals (Fig. 2c–d, *arrows*), whereas the remaining chromosomes harbored much weaker signals that were often difficult to detect. On the other hand, both accessions of *D. involucratus* ($2n = 22$) and *D. conchitae* ($2n = 22$) (both species from the *Daucus* II subclade) showed the fewest CentDc signals. In the *D. involucratus* accessions, CentDc repeats hybridized to two chromosome pairs, occupying centromeric regions (Fig. 2e–f), while *D. conchitae* produced CentDc signals on one chromosome pair (Fig. 2g). In the latter, the signals were difficult to score as centromeric; thus, we performed FISH to meiotic chromosomes of that species (see below).

Interestingly, CentDc signals were also found on four chromosome pairs of the outgroup species, *Astrodaucus littoralis* (Fig. 2h), suggesting the ancestral status of this repeat. We were, however, not able to determine whether the signals were centromeric. No signals were observed in the other outgroup species.

To confirm the centromeric position of the CentDc repeats or to visualize them at a greater resolution, FISH was performed on both meiotic chromosomes and chromosomes in mitotic anaphase of selected accessions (Additional file 1: Fig. S1). In some cases, depending on the degree of chromatin condensation, CentDc signals coincided with cytologically recognizable heterochromatic knobs on pachytene chromosomes. The results also showed that at meiotic metaphase I and mitotic anaphase, the signals were located at the most poleward positions, confirming the centromeric specificity of these repeats.

Karyotype analysis

Accessions selected for detailed karyotype analysis were those that (1) produced CentDc signals in the centromeric regions of all chromosomes and (2) had metaphase spreads containing only well-condensed chromosomes with clearly defined boundaries. The only exception was *Orlaya daucoides*, which, despite being FISH-negative, had chromosomes with a distinct primary constriction (Fig. 2o); therefore, it was also subjected to karyotyping. Although five other accessions met the first criterion (*D. aureus* [PI 295854], *D. carota* subsp. *maximus* [Ames 26408], *D. pumilus* [PI 662301], *D. pusillus* [PI 349267], *D. sahariensis* [Ames 29096]), we failed to obtain a sufficient number of good quality metaphase spreads (the second criterion); hence, they were excluded from karyotyping.

Table 1 List of *Daucus* accessions and related species (outgroups) used in this study

Taxon[a]	2*n*	Seed source[b]/Accession no.[c]	Country of origin
Daucus I subclade			
D. aureus	22	USDA/PI 295854	Israel
D. aureus	22	USDA/PI 319403	Israel
D. carota subsp. *capillifolius*	18	USDA/PI 279764	Libya
D. carota subsp. *capillifolius*	18	USDA/Ames 30198	Tunisia
D. carota subsp. *carota*	18	USDA/PI 274297	Pakistan
D. carota subsp. *carota*	18	USDA/PI 478369	China
D. carota subsp. *carota*	18	USDA/PI 478861	France
D. carota subsp. *carota*	18	USDA/PI 652393	Turkey
D. carota subsp. *gummifer*	18	USDA/PI 478883	France
D. carota subsp. *gummifer*	18	USDA/Ames 26383	Portugal
D. carota subsp. *maximus*	18	USDA/Ames 26408	Portugal
D. carota subsp. *sativus*	18	RZ/DH1*	The Netherlands
D. carota subsp. *sativus*	18	Commercial/'Dolanka'**	Poland
D. carota subsp. *sativus*	18	Commercial/'Amsterdam'**	Poland
D. crinitus	22	USDA/PI 652414	Portugal
D. muricatus	22	USDA/PI 295863	Spain
D. muricatus	22	USDA/Ames 29090	Tunisia
D. pumilus	16	USDA/PI 662301	Tunisia
D. rouyi	20	USDA/PI 674284	Tunisia
D. sahariensis	18	USDA/Ames 29096	Tunisia
D. sahariensis	18	USDA/Ames 29097	Tunisia
D. syrticus	18	USDA/Ames 29108	Tunisia
Daucus II subclade			
D. conchitae	22	USDA/Ames 25835	Turkey
D. glochidiatus	44	USDA/PI 285038	Australia
D. involucratus	22	USDA/PI 652332	Greece
D. involucratus	22	USDA/PI 652355	Turkey
D. littoralis	20	USDA/PI 295857	Israel
D. pusillus	22	USDA/PI 349267	Uruguay
Outgroups			
Ammi visnaga	20	IPK/AMMI 25	Germany
Astrodaucus littoralis	20	USDA/PI 277064	Azerbaijan
Caucalis platycarpos	20	USDA/PI 649446	Germany
Orlaya daucoides	16	USDA/PI 649477	Turkey
Torilis arvensis	12	USDA/PI 649391	Syria
T. arvensis	12	USDA/PI 649394	Turkey

[a] Taxonomic classification according to [25, 26]
[b] *IPK,* Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany; *RZ,* Rijk Zwaan vegetable breeding company, Lier, the Netherlands; *USDA,* USDA-ARS North Central Regional Plant Introduction Station (NCRPIS), Ames, Iowa, USA
[c] *Ames*, Ames numbers are assigned to carrots and other Apiaceae maintained at the NCRPIS; *PI*, USDA Plant Introduction numbers are permanent numbers assigned to germplasm accessions in the National Plant Germplasm System (NPGS); (*) *DH1*, a doubled haploid orange Nantes type carrot breeding line; (**) carrot cultivars

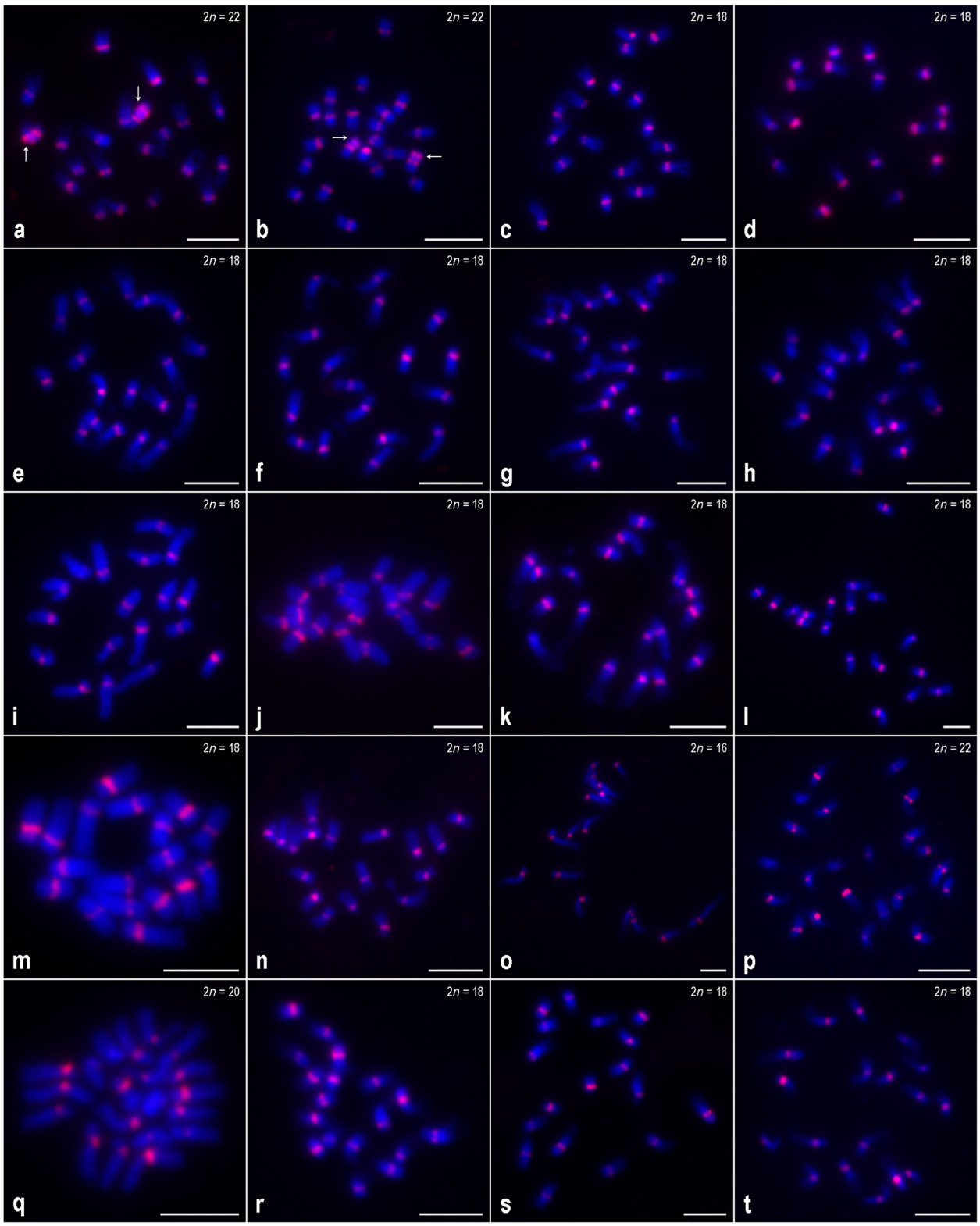

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 FISH mapping of the CentDc probe (red signals) to metaphase chromosomes of *Daucus* taxa. **a** *D. aureus* [PI 295854]; **b** *D. aureus* [PI 319403]; **c** *D. carota* subsp. *capillifolius* [PI 279764]; **d** subsp. *capillifolius* [Ames 30198]; **e** subsp. *carota* [PI 274297]; **f** subsp. *carota* [PI 478369]; **g** subsp. *carota* [PI 478861]; **h** subsp. *carota* [PI 652393]; **i** subsp. *gummifer* [PI 478883]; **j** subsp. *gummifer* [Ames 26383]; **k** subsp. *maximus*; **l** subsp. *sativus* ('Amsterdam'); **m** subsp. *sativus* ('Dolanka'); **n** subsp. *sativus* (DH1); **o** *D. pumilus*; **p** *D. pusillus*; **q** *D. rouyi*; **r** *D. sahariensis* [Ames 29096]; **s** *D. sahariensis* [Ames 29097]; **t** *D. syrticus*. *Arrows* in (**a**) and (**b**) indicate additional CentDc signals in the interstitial regions of the long arms of the chromosomes. Scale bar = 5 μm

The detailed karyotype features of the 16 karyotyped accessions (representing 9 taxa) are summarized in Table 2. The mean haploid idiograms of each accession are shown in Fig. 3.

Among the studied accessions, *O. daucoides* had the highest total haploid chromosome length (THCL); however, considering only the genus *Daucus*, the highest THCL was found in *D. rouyi*, while *D. carota* subsp. *capillifolius* [Ames 30198] had the lowest value of this parameter. The longest chromosome was also observed in *O. daucoides*, but in the genus *Daucus*, the longest chromosome occurred in *D. carota* subsp. *gummifer* [PI 478883], which was three times longer than the shortest chromosome that was found in *D. aureus*. The accessions differed in their haploid karyotype formula (KF), even within the same taxa, except *D. carota* subsp. *capillifolius*, whose both accessions shared the same KF. The karyotypes were composed of metacentric, submetacentric and subtelocentric chromosomes, with submetacentric being the most common form of chromosomes, representing 72.6% of all chromosomes.

Among the genus *Daucus*, *D. sahariensis* and *D. rouyi* exhibited the highest interchromosomal asymmetry, as evidenced by CV_{CL} values, while *D. carota* subsp. *sativus* (DH1) was found to have the most symmetrical karyotype in this regard. Moreover, *D. rouyi* also showed the highest intrachromosomal asymmetry, as indicated by the M_{CA} value, whereas *D. syrticus* had the lowest value of this parameter. CV_{CI} values showed that *D. carota* subsp. *carota* [PI 478861] had the most heterogeneous karyotype in terms of centromere position, while the karyotype of *D. syrticus* was the most homogeneous in this regard. However, when including *O. daucoides* (outgroup), this species was characterized by the most asymmetrical karyotype in terms of interchromosomal asymmetry, but at the same time, it showed the lowest intrachromosomal asymmetry of all karyotyped accessions. Relationships among the examined accessions based on the asymmetry indices are illustrated in Fig. 4. According to the classification of Stebbins [40], the karyotyped accessions were pooled into two classes, namely 2A and 3A, with a predominance of the 3A class (representing 75%).

The UPGMA dendrogram based on six karyological parameters divided the 16 accessions into three major clusters at a Euclidean distance of 12.5, with a cophenetic correlation of 0.92 (Fig. 5). The first cluster, represented by *O. daucoides*, was separate, as a distinct outgroup, forming an independent branch. In the second cluster, *D. syrticus* and *D. aureus* were grouped together. The third cluster was subdivided into two subclusters, one of which comprised *D. sahariensis* and all *D. carota* subspecies, while the other one included only *D. rouyi*.

Karyological relationships among the studied accessions revealed by PCoA are illustrated in Fig. 6. The results indicated that the first two principal coordinates explained 74% of the total variation. The PCoA scatter plot showed that all *D. carota* subspecies tended to cluster together, while three wild *Daucus* species, namely *D. sahariensis*, *D. syrticus*, and *D. rouyi*, were clearly separated from them. In contrast, *D. aureus* and *O. daucoides* occupied the most isolated positions, with *O. daucoides* being a distinct outgroup.

Discussion

Karyotype features, especially chromosome number, chromosome length, karyotype asymmetry, the number of rDNA sites, and other chromosomal markers, are of great use in plant taxonomy and evolutionary studies. Thus, comparative karyotype analyses have been broadly utilized to elucidate relationships among taxa (at different taxonomic levels), as well as to understand the trends in chromosome evolution [41–47]. Moreover, comparative cytogenetic studies have provided evidence for extensive chromosome rearrangements in several plant species, e.g., those belonging to the families Brassicaceae [48, 49], Solanaceae [50, 51], and Poaceae [52–54]. The differences in karyotypes between related species, i.e., the chromosome number, shape, and structure, are caused by the syntenic groups that are assembled in different combinations. For example, groups that are fused together in one species may be separated on different chromosomes in another, or may be duplicated, inverted, or lost [55].

Centromeres are the key regions of eukaryotic chromosomes and are essential for sister chromatid cohesion. Additionally, centromeres are the sites where spindle microtubules attach via the kinetochore complex, thereby ensuring the proper segregation of chromosomes during cell division. Microscopically, they are recognizable on metaphase chromosomes as the primary

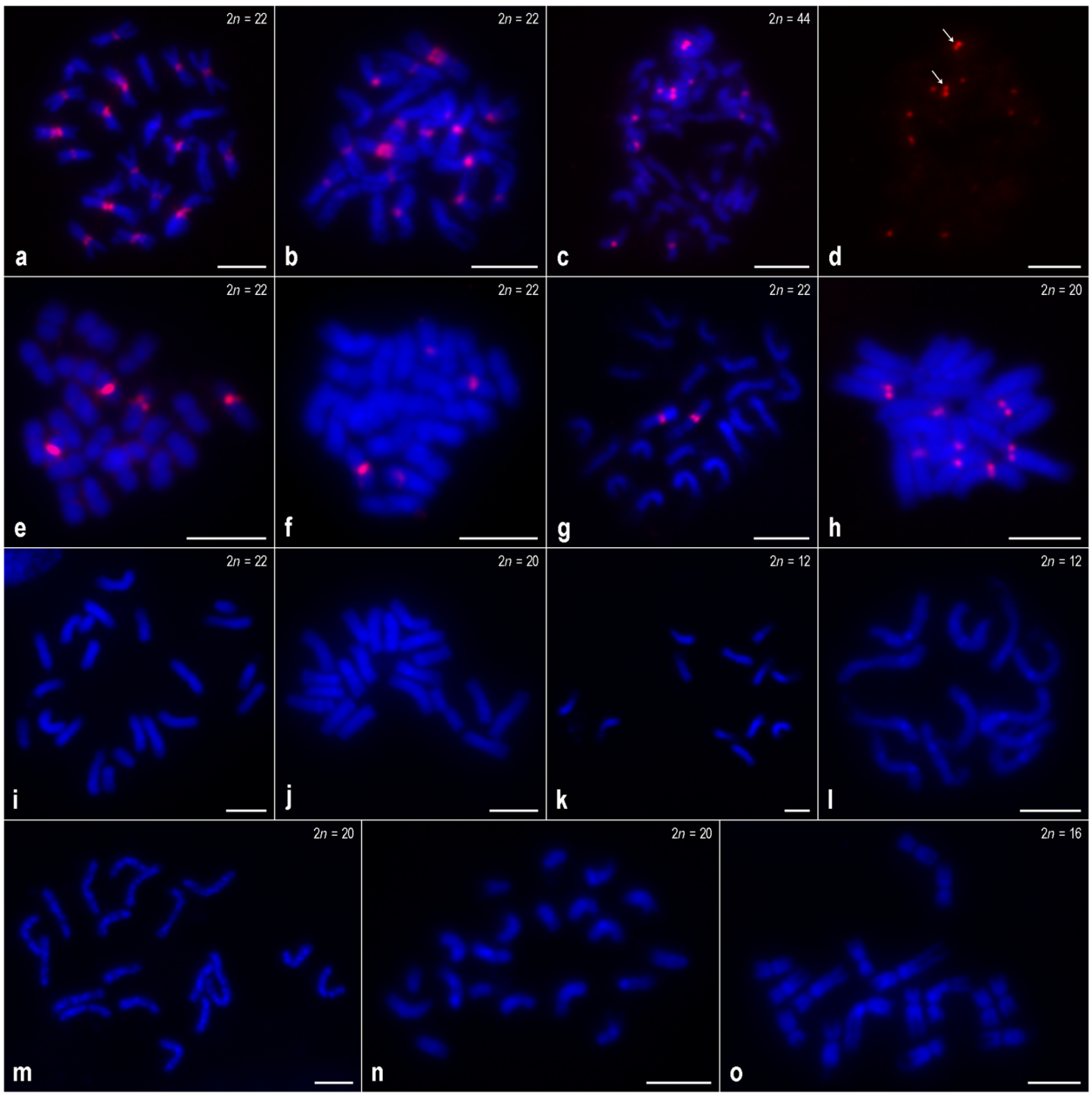

Fig. 2 FISH mapping of the CentDc probe (red signals) to metaphase chromosomes of *Daucus* taxa and related species. **a** *D. muricatus* [PI 295863]; **b** *D. muricatus* [Ames 29090]; **c** *D. glochidiatus*; **d** FISH signals from subpanel **c**, *arrows* indicate a chromosome pair with distinctly strong signals; **e** *D. involucratus* [PI 652332]; **f** *D. involucratus* [PI 652355]; **g** *D. conchitae*; **h** *Aastrodaucus littoralis*; **i** *D. crinitus*; **j** *D. littoralis*; **k** *Torilis arvensis* [PI 649391]; **l** *T. arvensis* [PI 649394]; **m** *Ammi visnaga*; **n** *Caucalis platycarpos*; **o** *Orlaya daucoides*. **i**–**o** These species did not produced CentDc signals after FISH. Scale bar = 5 μm

constrictions and mostly contain large arrays of highly repetitive satellite DNA and retrotransposons [56, 57]. Typically, the monomers of centromeric satellite repeats range from 150 to 180 bp in length, e.g., pAL1 in *Arabidopsis* [58, 59], CentO in rice [60], CentC in maize [61], MtRs in *Medicago truncatula* [62], CL1 repeat in radish [16], CmSat162 in melon [14], and So1 in sugarcane [63]; however, longer monomers have also been reported [63–69]. Although the functional role of centromeres is conserved among all eukaryotes, the sequences of centromeric DNA and kinetochore proteins are considerably variable and evolve rapidly, even in closely related species, which is known as the 'centromeric paradox' [65, 70, 71]. For example, centromeres of rice (*Oryza sativa*) chromosomes contain a 155-bp satellite repeat CentO [60], whereas several wild *Oryza* species lack this

Table 2 Karyotype features of the studied accessions

Taxon	Accession	2*n*	KF[a]	THCL[b] (μm)	CLR[c] (μm)	CV_{CL}[d]	CV_{CI}[e]	M_{CA}[f]	St[g]
Daucus aureus	PI 319403	22	1m + 10sm	23.50	1.54–2.89	17.85	10.23	36.89	3A
D. carota subsp. *capillifolius*	PI 279764	18	8sm + 1st	28.67	2.32–4.08	17.68	22.12	34.63	3A
D. carota subsp. *capillifolius*	Ames 30198	18	8sm + 1st	22.19	1.89–3.36	18.33	16.94	34.79	3A
D. carota subsp. *carota*	PI 274297	18	7sm + 2st	28.95	2.38–4.03	17.26	20.19	34.01	3A
D. carota subsp. *carota*	PI 478369	18	1m + 6sm + 2st	24.56	2.07–3.61	16.21	18.24	33.36	2A
D. carota subsp. *carota*	PI 478861	18	1m + 4sm + 4st	31.15	2.62–4.33	15.13	26.50	35.89	3A
D. carota subsp. *carota*	PI 652393	18	6sm + 3st	25.23	2.04–3.45	15.52	19.38	35.79	3A
D. carota subsp. *gummifer*	PI 478883	18	5sm + 4st	33.47	2.72–4.77	17.42	24.31	35.94	3A
D. carota subsp. *gummifer*	Ames 26383	18	1m + 5sm + 3st	28.32	2.39–4.20	17.41	18.45	34.06	2A
D. carota subsp. *sativus*	'Amsterdam'	18	7sm + 2st	29.86	2.55–4.16	16.04	24.37	35.73	3A
D. carota subsp. *sativus*	'Dolanka'	18	1m + 8sm	29.90	2.65–4.26	14.70	18.93	31.04	3A
D. carota subsp. *sativus*	DH1	18	8sm + 1st	28.08	2.42–3.91	14.38	19.65	34.45	3A
D. rouyi	PI 674284	20	2m + 8sm	35.50	2.45–5.12	20.41	14.47	38.74	3A
D. sahariensis	Ames 29097	18	2m + 7sm	27.97	2.24–4.20	20.46	13.68	32.28	3A
D. syrticus	Ames 29108	18	6m + 3sm	26.86	2.25–4.07	17.66	9.39	26.08	2A
Orlaya daucoides	PI 649477	16	2m + 6sm	35.61	3.27–7.73	30.47	12.16	20.55	2A

Note: The formulae of the above parameters (CV_{CL}, CV_{CI}, M_{CA}) and the karyotype symmetry classes of Stebbins are given in Additional file 2: Table S1 and Additional file 3: Table S2, respectively

[a] *KF*, haploid karyotype formula (*m*, metacentric; *sm*, submetacentric; *st*, subtelocentric)

[b] *THCL*, mean total haploid chromosome length

[c] *CLR*, Chromosome length range

[d] CV_{CL} , Coefficient of variation of chromosome length

[e] CV_{CI} , Coefficient of variation of the centromeric index

[f] M_{CA} , Mean centromeric asymmetry

[g] *St*, karyotype symmetry class according to Stebbins [40]

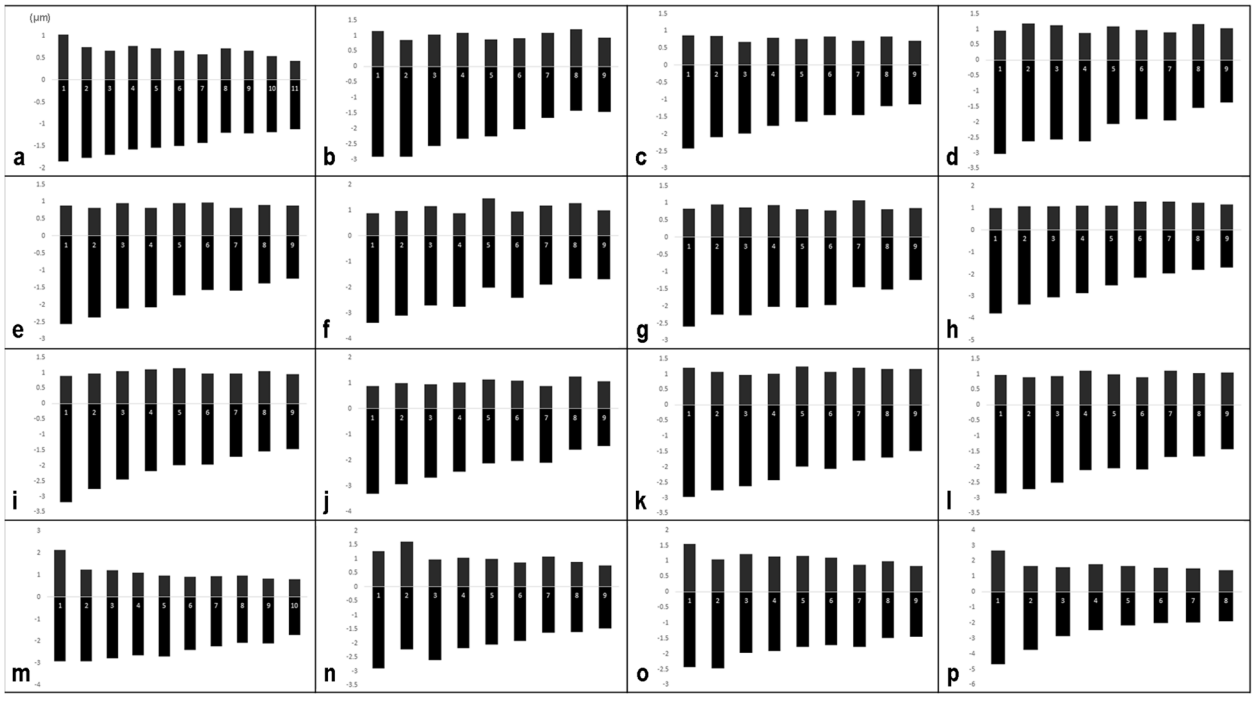

Fig. 3 The mean haploid idiograms of the 16 accessions of *Daucus* and *Orlaya daucoides*, which are listed in Table 2. **a** *D. aureus*; **b** *D. carota* subsp. *capillifolius* [PI 279764]; **c** subsp. *capillifolius* [Ames 30198]; **d** subsp. *carota* [PI 274297]; **e** subsp. *carota* [PI 478369]; **f** subsp. *carota* [PI 478861]; **g** subsp. *carota* [PI 652393]; **h** subsp. *gummifer* [PI 478883]; **i** subsp. *gummifer* [Ames 26383]; **j** subsp. *sativus* ('Amsterdam'); **k** subsp. *sativus* ('Dolanka'); **l** subsp. *sativus* (DH1); **m** *D. rouyi*; **n** *D. sahariensis*; **o** *D. syrticus*; **p** *O. daucoides*

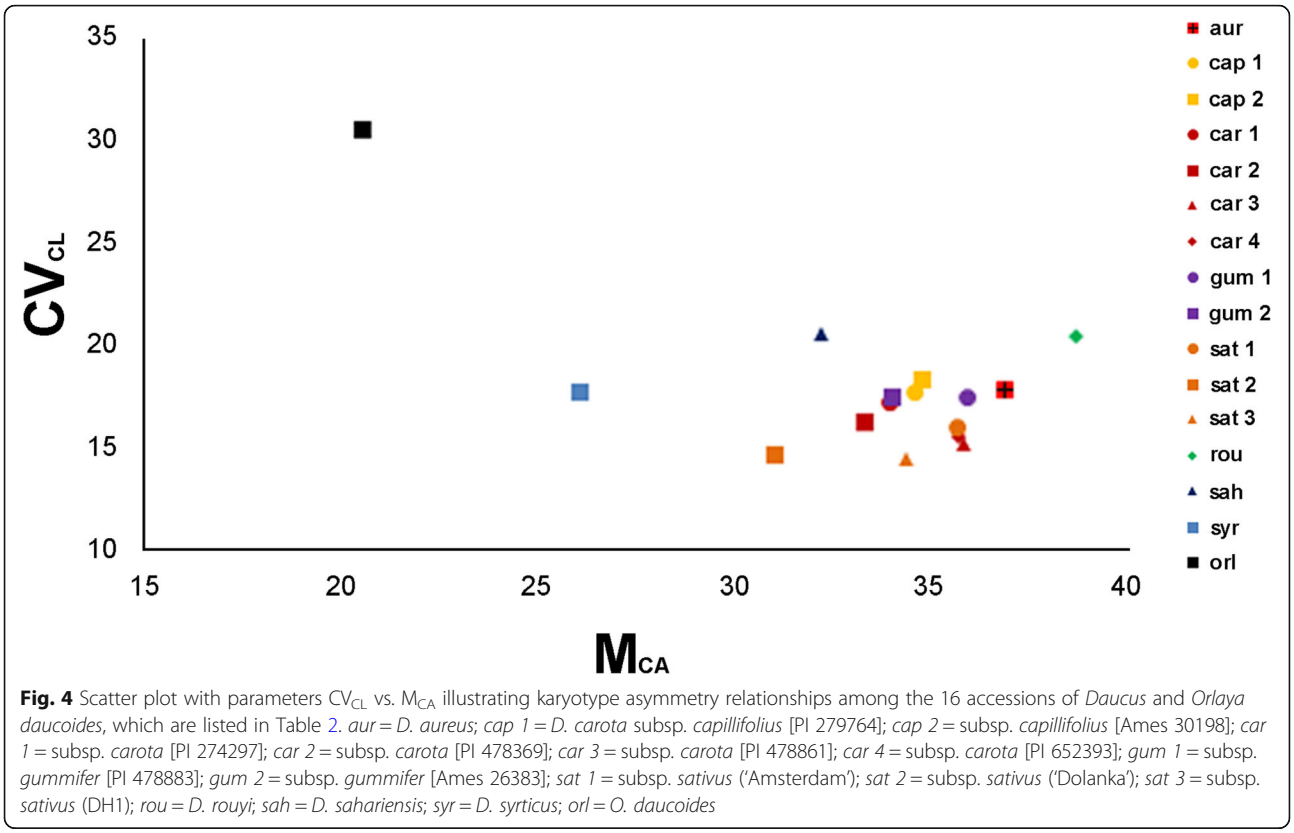

Fig. 4 Scatter plot with parameters CV_{CL} vs. M_{CA} illustrating karyotype asymmetry relationships among the 16 accessions of *Daucus* and *Orlaya daucoides*, which are listed in Table 2. *aur* = *D. aureus*; *cap 1* = *D. carota* subsp. *capillifolius* [PI 279764]; *cap 2* = subsp. *capillifolius* [Ames 30198]; *car 1* = subsp. *carota* [PI 274297]; *car 2* = subsp. *carota* [PI 478369]; *car 3* = subsp. *carota* [PI 478861]; *car 4* = subsp. *carota* [PI 652393]; *gum 1* = subsp. *gummifer* [PI 478883]; *gum 2* = subsp. *gummifer* [Ames 26383]; *sat 1* = subsp. *sativus* ('Amsterdam'); *sat 2* = subsp. *sativus* ('Dolanka'); *sat 3* = subsp. *sativus* (DH1); *rou* = *D. rouyi*; *sah* = *D. sahariensis*; *syr* = *D. syrticus*; *orl* = *O. daucoides*

sequence but instead contain different genome-specific centromeric satellite arrays [72, 73]. In potato (*Solanum tuberosum*), 12 centromeres show a large variation in terms of the structure and DNA composition, of which five centromeres lack satellite repeats but consist mainly of single- and low-copy sequences. In contrast, six potato centromeres contain megabase-sized arrays of satellite repeats, specific to individual centromeres; five of them appear to have emerged recently, since they were not found in the genomes of closely related *Solanum* species. In addition, most of these 'young' (newly emerged) centromeric repeats in potato were amplified from retrotransposon-related sequences [71]. Recently, Ávila Robledillo et al. [68] performed the largest study to date in terms of the number of related species investigated (14 species belonging to the legume tribe Fabeae) and newly centromeric satellites described. As a result, they found a great diversity of centromeric repeats within and between the analyzed Fabeae species. More recently, Huang et al. [63] discovered that some sugarcane centromeric satellites also exhibit high similarity with centromeric retrotransposons, indicating that they originated from these mobile elements. These repeats were flanked by direct repeats and formed extrachromosomal circular DNAs (eccDNAs). The retrotransposon-derived origin and the presence of eccDNAs elucidate how retrotransposons could evolve into centromeric satellites, providing new insights into the origin, formation pathways, and evolution of centromeric satellites in eukaryotes.

The original carrot centromeric repeat (CentDc) was isolated from BAC clone 004H08, which was initially selected for the *phytoene synthase 1* (*PSY1*) gene [39]. This BAC clone, as revealed by FISH, hybridized to the centromeric regions of all carrot chromosomes. Moreover, the FISH signals coincided with those produced by the carrot cot-1 DNA fraction, indicating that this BAC clone contained a dominant centromeric repeat [30]. As further evidenced by sequencing, the CentDc repeat unit of approximately 159 bp is composed of typically four 39–40-bp monomers (named A, B, C, and D) that vary slight in sequence, representing a higher-order repeat (HOR) structure [30, 33].

A comparative in silico analysis with some other *Daucus* species (from both *Daucus* I and II subclades) was also performed, revealing that the CentDc-like repeat represented the most abundant tandem repeat in the genomes of *D. syrticus* (named Ds-CL1) and *D. aureus* (named Da-CL1), varying, however, in terms of their structure. In *D. pusillus*, only the initial 40-bp monomer of its most abundant tandem repeat (named Dp-CL5) showed considerable similarity with monomer A of the original carrot CentDc element. These results indicate that these satellite families (CentDc, Ds-CL1, Da-CL1,

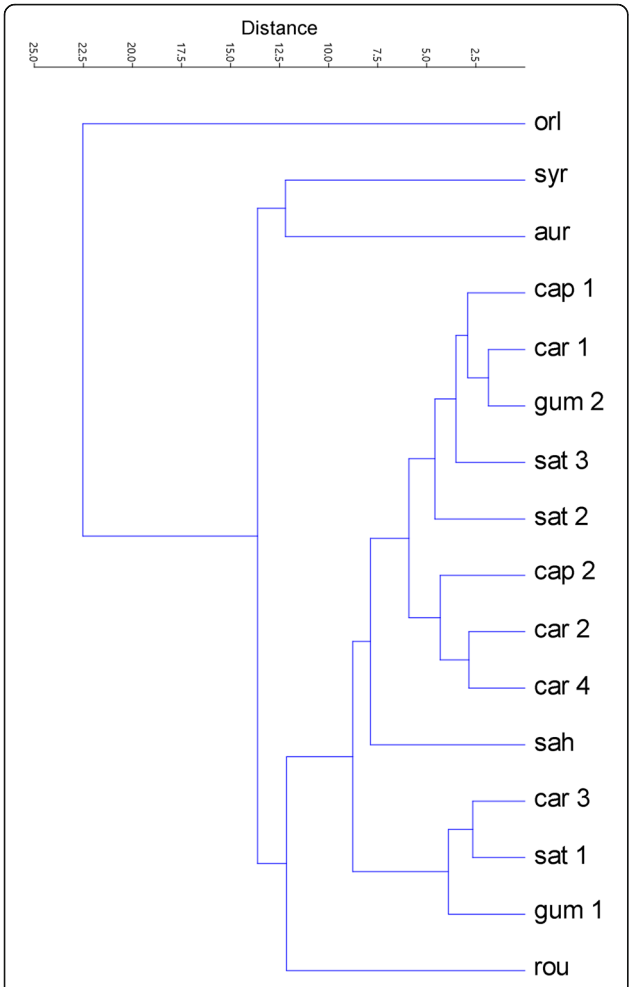

Fig. 5 UPGMA dendrogram based on six quantitative parameters (*x*, 2*n*, THCL, M_{CA}, CV_{CL}, CV_{CI}) showing the karyological relationships of the 16 accessions of *Daucus* and *Orlaya daucoides*, which are listed in Table 2. *aur* = *D. aureus*; *cap 1* = *D. carota* subsp. *capillifolius* [PI 279764]; *cap 2* = subsp. *capillifolius* [Ames 30198]; *car 1* = subsp. *carota* [PI 274297]; *car 2* = subsp. *carota* [PI 478369]; *car 3* = subsp. *carota* [PI 478861]; *car 4* = subsp. *carota* [PI 652393]; *gum 1* = subsp. *gummifer* [PI 478883]; *gum 2* = subsp. *gummifer* [Ames 26383]; *sat 1* = subsp. *sativus* ('Amsterdam'); *sat 2* = subsp. *sativus* ('Dolanka'); *sat 3* = subsp. *sativus* (DH1); *rou* = *D. rouyi*; *sah* = *D. sahariensis*; *syr* = *D. syrticus*; *orl* = *O. daucoides*

and Dp-CL5) share a common evolutionary origin, predating the divergence of the two *Daucus* subclades. However, in two other species, *D. guttatus* and *D. littoralis*, CentDc-like sequences were not found, which was further confirmed by FISH [33].

To study the evolution of carrot centromeric satellite repeats, for comparative FISH analysis, we selected a number of *Daucus* taxa, including some cultivated carrots and several wild species and subspecies, as well as some closely related non-*Daucus* species. The studied accessions differed in terms of their chromosome number, geographical distribution, and phylogenetic position. As a result, we found that CentDc repeats were present in the genomes of several taxa of both *Daucus* subclades and one non-*Daucus* outgroup species (*Astrodaucus littoralis*), which indicates the ancient nature of CentDc, confirming the previous conclusion of Iorizzo et al. [33].

Moreover, our findings were also consistent with the results of the above-mentioned in silico analysis by Iorizzo et al. [33]. Out of the five species examined by the authors, we included four in our comparative FISH study, i.e., the same accessions of *D. aureus*, *D. littoralis*, *D. pusillus*, and *D. syrticus*, confirming the presence of CentDc-like sequences in the genomes of *D. aureus*, *D. pusillus*, and *D. syrticus* as well as the absence of these sequences in *D. littoralis*.

Although we found CentDc repeats in the genome of one outgroup species, this evidence is not enough to allow assumptions on the phylogeny of this species. With regard to the phylogenetic position of the outgroup species, according to Arbizu et al. [25], who examined 107 accessions of *Daucus* (92 accessions) and non-*Daucus* (15 accessions) taxa using DNA sequences of 94 nuclear orthologs, among the analyzed outgroup species, *Orlaya daucoides* and *O. daucorlaya* are sister to *Daucus*, whereas *Astrodaucus littoralis*, *Caucalis platycarpos*, *Turgenia latifolia*, *Torilis leptophylla*, *T. arvensis*, and *T. nodosa* are sister to all other examined taxa, and *Ammi visnaga* and *Oenanthe virgata* are sister to all of the above.

Although different *Daucus* taxa had various numbers and intensities of FISH signals, the CentDc repeats maintained the consistency of their centromeric localization. Such consistency in terms of chromosomal distribution of different satellites among closely related species has also been reported, e.g., in *Saccharum* (the centromeric satellite So1) [63] or radish and *Brassica* species (the subtelomeric satellite CL25) [16]. In the case of FISH-negative taxa, the absence of the CentDc repeats suggests that they may have been lost or replaced by different centromeric satellites or that their copy number was too low to be detected by means of molecular cytogenetics.

It should be noted, however, that, in this study, we used a 36-nucleotide FISH probe [31] based on the consensus sequence corresponding to a subrepeat of the original CentDc repeat [30, 39]. In the FISH-positive *Daucus* taxa, we determined the centromeric localization of these repeats using metaphase spreads with distinct primary constrictions (Additional file 4: Fig. S2), which is often challenging in *Daucus* and other species with small chromosomes, especially when the chromosomes are highly condensed. Although we also conducted FISH on both meiotic chromosomes and chromosomes in mitotic anaphase of selected accessions, it is essential to

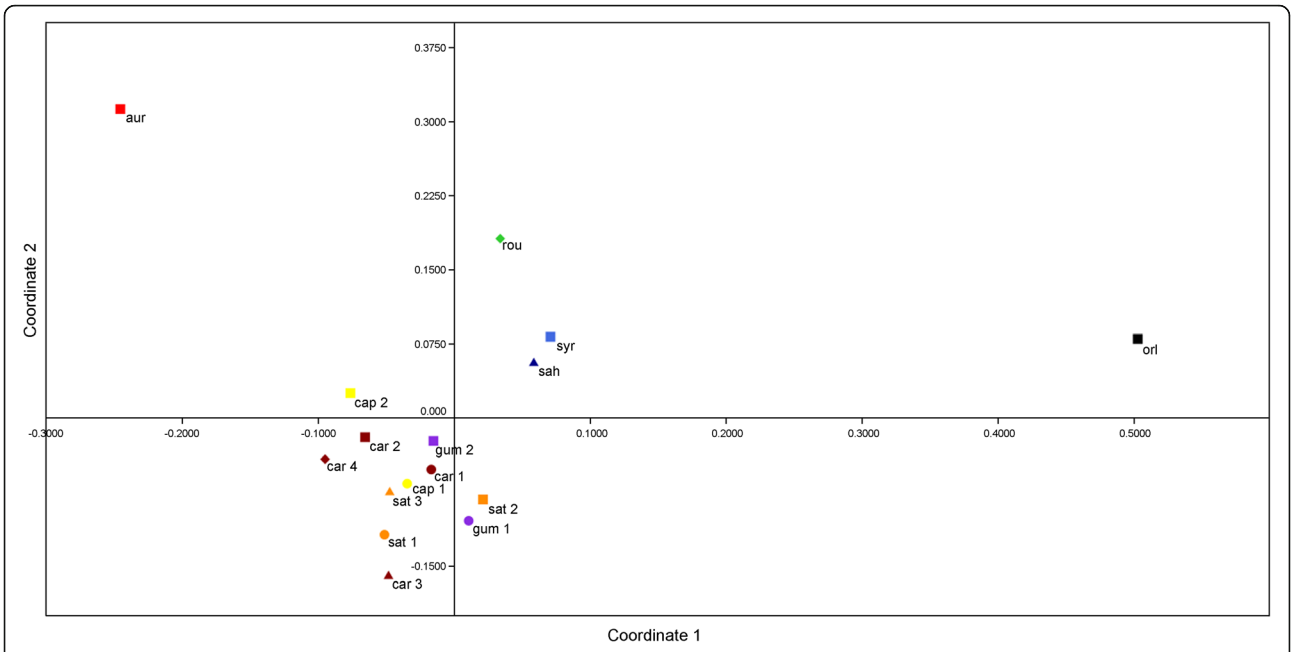

Fig. 6 PCoA based on six quantitative parameters (*x*, 2*n*, THCL, M_{CA}, CV_{CL}, CV_{CI}) for the 16 accessions of *Daucus* and *Orlaya daucoides*, which are listed in Table 2. *aur* = *D. aureus*; *cap 1* = *D. carota* subsp. *capillifolius* [PI 279764]; *cap 2* = subsp. *capillifolius* [Ames 30198]; *car 1* = subsp. *carota* [PI 274297]; *car 2* = subsp. *carota* [PI 478369]; *car 3* = subsp. *carota* [PI 478861]; *car 4* = subsp. *carota* [PI 652393]; *gum 1* = subsp. *gummifer* [PI 478883]; *gum 2* = subsp. *gummifer* [Ames 26383]; *sat 1* = subsp. *sativus* ('Amsterdam'); *sat 2* = subsp. *sativus* ('Dolanka'); *sat 3* = subsp. *sativus* (DH1); *rou* = *D. rouyi*; *sah* = *D. sahariensis*; *syr* = *D. syrticus*; *orl* = *O. daucoides*

differentiate sequences truly associated with centromeric chromatin from other repetitive sequences. Thus, to ascertain the centromeric localization of CentDc repeats and comprehensively investigate the repeat composition of *Daucus* centromeres, it would be necessary to perform chromatin immunoprecipitation (ChIP) using antibodies against centromeric proteins (anti-CENH3), followed by sequencing of the immunoprecipitated DNA (ChIP-Seq). In addition, immunofluorescence experiments using an antibody against the carrot CENH3 protein (anti-DcCENH3 antibody) were performed in carrot and *D. glochidiatus* [74]. Combined localization of CENH3 and CentDc (immuno-FISH) has not yet been applied; however, such an approach should be considered in future research.

Of the FISH-positive taxa, the unique hybridization patterns of CentDc repeats in *D. aureus*, *D. conchitae*, *D. glochidiatus*, *D. involucratus*, and *D. muricatus* suggest that the CentDc probe may be used as a marker for the identification of these species. Moreover, in the case of *D. aureus*, *D. involucratus*, and *D. muricatus*, these findings were confirmed using different accessions of these species, which – except for *D. aureus* accessions – originated from different countries (see Table 1). However, to ascertain the species-specific FISH patterns of CentDc repeats for these species, it is necessary to analyze the remaining taxa from the genus *Daucus* that were not included in our study.

The haploid chromosome numbers of the majority of species within *Daucus* range between $n = 8$ and 11. One of the first reports on the somatic chromosome number of carrot ($2n = 18$) was published by Lindenbein [75], which was further confirmed by Sharma and Ghosh [35]. Only four other species, namely *D. annuus*, *D. insularis*, *D. sahariensis*, and *D. syrticus*, also have nine pairs of chromosomes [76, 77]. Most *Daucus* species are diploids with $2n = 20$ or 22, yet some polyploids exist as well, i.e., tetraploid *D. glochidiatus*, *D. incognitus*, *D. melananthos*, and *D. pedunculatus* ($2n = 44$) and hexaploid *D. montanus* ($2n = 66$) [78–80]. Here, we confirmed the previous chromosome counts for the investigated species; however, in the case of *D. conchitae*, to the best of our knowledge, we provided data on its somatic chromosome number for the first time.

Chromosome measurements may be precise only when the chromosomes are fully condensed and their boundaries are well defined. Thus, when analyzing chromosome spreads, it is crucial to exclude the ones containing either metaphase chromosomes that have not reached their maximum degree of condensation or prometaphase chromosomes. Moreover, chromosomes should have morphologically distinct primary constrictions; otherwise, it is difficult to determine the length of chromosome arms and, consequently, to calculate chromosomal parameters, such as centromeric index and karyotype asymmetry indices.

Karyotype asymmetry is an important karyotype character reflecting the general morphology of plant chromosomes and is thus widely used in comparative cytotaxonomy. A symmetrical karyotype comprises predominantly metacentric and submetacentric chromosomes of approximately equal size. Increased asymmetry may be caused either by the shifts in centromere position towards the telomeres (intrachromosomal) or by structural changes in chromatin (additions or deletions) that involve some chromosomes, leading to differences in the relative size between the chromosomes of the complement (interchromosomal) [41, 81, 82]. To date, several parameters and indices describing karyotype asymmetry have been proposed, including the quali-quantitative one proposed by Stebbins [40], as well as quantitative indices, of which Rec [83], A_2 [84], R ratio [85], CV_{CL} [81] are measures of interchromosomal asymmetry, and TF% [86], AsK% [87], AsI% [88], Syi [83], A_1 [84], A [89], CV_{CI} [81], and M_{CA} [90] characterize intrachromosomal asymmetry. Many of these parameters are, however, outdated and statistically incorrect, yet they are still widely used by a number of researchers [81, 90].

Since it is crucial to use only the parameters with a solid statistical basis for comparing karyotypes and reconstructing karyological relationships among taxa, here, we applied the methodology proposed by Peruzzi and Altınordu [91], considering six quantitative parameters (*x*, 2*n*, THCL, M_{CA}, CV_{CL}, CV_{CI}) and subjecting them to principal coordinate analysis (PCoA), which is – thus far – the most legitimate approach to use. Our results showed that PCoA with these parameters was indeed a good way to establish the karyological relationships among taxa, as it clearly separated the wild *Daucus* species from the closely clustered *D. carota* subspecies. However, we observed some karyotypic variations between different accessions belonging to the same subspecies, which is especially noticeable in the UPGMA dendrogram, as they were placed into separate subsubclusters. Moreover, the haploid karyotype formulae, to a large extent, also differed. This intrasubspecific diversity might be attributed to the different geographical distribution of these accessions, where different ecological, climatic, and altitude conditions occur. Similar observations were also made in some other taxa, e.g., *Dianthus* spp. [92] and *Zygophyllum fabago* [93], of which various geographically distant populations were sampled.

In terms of the Stebbins' system, the karyotyped accessions were placed in 2A and 3A classes, indicating that these accessions have relatively symmetrical karyotypes (see Additional file 3: Table S2), and – from the evolutionary point of view – they are considered as primitive in this system [40].

Considering the karyotype of carrot, different researchers obtained different results in terms of karyotype formulae. Sharma and Ghosh [35], Sharma and Bhattacharyya [36], and Iovene et al. [29] observed a predominance of chromosomes with median and submedian primary constrictions for several cultivated forms of carrot. In contrast, our results resemble those described by Schrader et al. [38] and Nowicka et al. [32], who observed more asymmetrical karyotypes for different cultivated carrot forms. However, Schrader et al. [38] used Giemsa C-banded prometaphase chromosomes and did not specify the chromosome classification; on the other hand, works by Sharma and Ghosh [35] and Sharma and Bhattacharyya [36] had been published before Levan et al. [94] proposed a new (commonly used today) classification system; hence, these results are difficult to compare. Nevertheless, the observed discrepancies may be due to several reasons, including the line/cultivar/accession used, chromosome preparation methodology, or environmental conditions (e.g., climate, altitude). The latter may act as mutagenic factors, leading to changes in chromosome structure (deletions, additions), or may induce the activity of transposable elements; both of which cause variations in DNA content and, consequently, karyotype structure among accessions within a given species [95, 96]. However, the results on the effect of environmental factors on plant genomes have been inconclusive so far [95, 97, 98].

From the perspective of increasing human population, the need to increase carrot and other crops' productivity is an actual challenge for researchers, inducing the development of breeding programs, that aim at obtaining new varieties that may be higher yielding, disease-resistant, or adapted to unfavorable conditions, especially in light of climate change and the alteration of natural ecosystems by human activities. The wild *Daucus* relatives may, therefore, play a significant role in the improvement of modern agriculture, providing genes that could be beneficial for breeding purposes, e.g., in adaptation to biotic and abiotic stresses, or climate change. In this context, a better understanding of the evolutionary relationships within the genus *Daucus* will contribute to future crop improvement programs [25, 79, 99].

Conclusions

In this study, we determined the genomic organization of carrot centromeric repeats (CentDc) in 26 accessions of *Daucus* (belonging to both *Daucus* I and II subclades) and one accession of a closely related species. We showed that CentDc elements were present in the centromeric regions of all chromosomes of 20 accessions, representing 11 taxa, and thus can be used as centromere-specific cytogenetic markers. In the other *Daucus* taxa, the number of chromosome pairs with

CentDc signals varied depending on the species, yet their centromeric localization was conserved. The presence of the CentDc repeats in the genomes of taxa belonging to both *Daucus* subclades and one outgroup species indicates the ancestral status of the repeat. In addition, we demonstrated the great usefulness of combining molecular cytogenetics with traditional chromosome measurements to study inter- and intraspecific karyological relationships among *Daucus* taxa.

Our observations provide useful information for further evolutionary, cytotaxonomic, and phylogenetic research on the genus *Daucus* and may contribute to a better understanding of the dynamic evolution of centromeric satellites in plants.

Methods

Plant material and chromosome preparation

In total, 34 accessions representing 22 taxa (species or subspecies), including 28 accessions from *Daucus* genus and 6 from closely related non-*Daucus* species, were selected for comparative FISH analysis (Table 1). Among the *Daucus* accessions, 12 were subspecies of *D. carota* (including one breeding line and two carrot cultivars) and 16 were from wild species belonging to *Daucus* subclades I and II. Seeds of all wild accessions were provided by the USDA-ARS North Central Regional Plant Introduction Station (Ames, Iowa, USA) and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK; Gatersleben, Germany), whereas the seeds of cultivated carrot were obtained either from the collections of the Department of Plant Biology and Biotechnology, University of Agriculture in Krakow (Krakow, Poland) or purchased from commercial sources.

The seeds were germinated either in soil-filled pots and grown under greenhouse conditions at 18 °C with a 16/8 h (light/dark) photoperiod or on moist filter paper in Petri dishes at 18 °C in the dark. Metaphase spreads were prepared from meristem root tip cells according to Nowicka et al. [31]. Root tips, approximately 1–2 cm in length, were collected from young plants or seedlings, pre-treated with 2 mM 8-hydroxyquinoline (Duchefa, Haarlem, the Netherlands) for 3.5 h at room temperature in the dark, and fixed in a freshly prepared mixture of methanol and glacial acetic acid (3:1, v/v) for at least 48 h. Meristems were then excised and digested in a mixture of 4% (w/v) cellulase Onozuka R-10. (Duchefa) and 2% (w/v) pectolyase Y-23 (Duchefa) in distilled water (pH 4.8) at 37 °C for 30–40 min. After digestion, the meristems were washed twice in distilled water, refixed in fixative, then macerated on a glass slide (one meristems per slide) using fine-pointed forceps, and flame-dried.

For meiotic preparations, flower buds at early stages of development were collected from greenhouse-grown plants of selected accessions and fixed in a freshly prepared mixture of ethanol and glacial acetic acid (3:1, v/v) for at least 48 h. After fixation, the flower buds were washed in a 10 mM citrate buffer (pH 4.8), and anthers were excised from the buds under a Leica S6D dissecting microscope (Leica Microsystems, Heerbrugg, Switzerland). The anthers were then digested in a mixture of 4% (w/v) cellulase Onozuka R-23, 2% (w/v) pectolyase Y-23, and 0.1% (w/v) cytohelicase (Sigma-Aldrich, St. Louis, USA) in 10 mM citrate buffer (pH 4.8) at 37 °C for 70–120 min. The digested anthers were washed twice in distilled water, refixed in fixative, then macerated on a glass slide (two anthers per slide) using fine-pointed forceps, and flame-dried.

To obtain cells in anaphase, a portion of the roots was not subjected to the treatment with 8-hydroxyquinoline but instead was fixed directly after collecting.

DNA probe and fluorescence in situ hybridization

For comparative FISH mapping, we used a 36-nucleotide probe with the following sequence: 5′–ACTCGTTTGAAGTTGGAAACAACTTGTAGCTTCATT–3′ [31], which was designed and directly labeled with cyanine-5 (Cy5) at the 5′-end during synthesis by Genomed (Warsaw, Poland). The probe was based on the consensus sequence corresponding to a subrepeat of the previously described carrot centromeric repeat, named CentDc [30, 39]. Further, in this paper, we also refer to this 36-nucleotide sequence as CentDc.

The FISH procedure was carried out according to Czernicka et al. [100] with minor modifications. A hybridization mixture containing 50% (v/v) deionized formamide, 10% (w/v) dextran sulfate (Sigma-Aldrich), 2× SSC (0.3 M NaCl, 0.03 M $Na_3C_6H_5O_7$; pH 7.0), and 50 ng μL^{-1} probe was denatured at 90 °C for 6 min, and instantly quenched in ice. The slides were denatured in 70% formamide/2× SSC at 80 °C for 1.5 min, immediately dehydrated in a graded ethanol series (70% ice-cold, 90 and 100% for 5 min each), and air-dried. The hybridization mixture was then applied to the slides, covered with a cover glass, sealed with rubber cement, and allowed to hybridize overnight at 37 °C in a humid chamber. After post-hybridization washes [2× SSC for 5 min, 2× SSC at 42 °C for 10 min, 2× SSC for 5 min, 1× PBS (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; pH 7.4) for 5 min], chromosomes were counterstained with 1 μg mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI) mounting medium (ProLong® Gold Antifade Mountant with DAPI; Thermo Fisher Scientific, Invitrogen™, Carlsbad, USA), and covered with a cover glass. For each accession, at least 3–5 plants were examined and thereby at least three independent FISH experiments per accession were performed.

The slides were examined under an Axio Imager.M2 fluorescence microscope (Carl Zeiss, Göttingen, Germany) equipped with the appropriate filter sets for

DAPI (Zeiss filter set 02: λ_{ex} = 365 nm, λ_{em} > 420 nm) and Cy5 (Zeiss filter set 50: λ_{ex} = 640/30 nm, λ_{em} = 690/50 nm). The images were captured using a BV MV camera (Applied Spectral Imaging, Edingen-Neckarhausen, Germany) and Case Data Manager 6.0 software (Applied Spectral Imaging) and processed with FISHView® (Applied Spectral Imaging).

Karyotype analysis

For karyotype analysis, 16 accessions, representing 9 taxa, were selected (for selection criteria, see 'Results'). For each accession, 4–10 well-spread mitotic metaphase plates were examined. Karyotypic parameters, including total haploid chromosome length (THCL) and chromosome length range (CLR), were determined using KaryoType 2.0 software [101]. Nomenclature used for the karyotype description followed that of Levan et al. [94]. To estimate karyotype asymmetry, the following karyotype asymmetry indices were used: CV_{CL} = coefficient of variation of chromosome length, CV_{CI} = coefficient of variation of centromeric index [81], and M_{CA} = mean centromeric asymmetry [90]; the formulae of these parameters are given in Additional file 2: Table S1. In addition, the accessions were categorized according to the karyotype symmetry classification of Stebbins [40] (Additional file 3: Table S2). For each karyotyped accession, a mean haploid idiogram was constructed by arranging the chromosomes in order of decreasing length.

To visualize karyotype asymmetry relationships among the studied accessions, a bidimensional scatter plot with parameters CV_{CL} vs. M_{CA} was drawn. To determine the karyological relationships among accessions, an unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis with Euclidean distance and principal coordinate analysis (PCoA) using Gower's similarity coefficient were performed based on six quantitative parameters (*x*, 2*n*, THCL, M_{CA}, CV_{CL}, CV_{CI}), as proposed by Peruzzi and Altınordu [91]. Statistical analyses were performed using Past 3.22 software [102], and the UPGMA-based dendrogram and PCoA scatter plot were generated.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-021-07853-2.

Additional file 1: Fig. S1. FISH mapping of CentDc probe (red signals) to meiotic chromosomes (**a–g**) and chromosomes in mitotic anaphase (**h**) of selected *Daucus* accessions. **a** Pachytene chromosomes of *D. carota* subsp. *sativus* ('Dolanka'); **b** DAPI-stained chromosomes from subpanel (**a**) that were digitally converted to a black-and-white image depicting cytologically recognizable heterochromatic knobs (*asterisks*), which CentDc signals coincide with, *arrowheads* indicate poorly visible knobs; **c** diakinesis chromosomes of Dolanka; **d** diakinesis and **e** metaphase I chromosomes of *D. aureus* [PI 319403], *arrows* indicate the chromosome pairs with additional CentDc signals; **f** pachytene chromosomes of *D. muricatus* [PI 295863], *arrow* indicates CentDc signals in the pericentromeric regions of one chromosome pair; **g** diakinesis chromosomes of *D. conchitae*, arrows indicate signals located at the most poleward positions of the chromosomes; **h** *D. pumilus*, CentDc signals located at the most poleward positions of the chromosomes in mitotic anaphase. Scale bar = 5 μm

Additional file 2: Table S1. Karyological parameters used in this study.

Additional file 3: Table S2. The classification of karyotypes in relation to their degree of asymmetry according to Stebbins (1971).

Additional file 4: Fig. S2. FISH mapping of CentDc probe (red signals) to the centromeric regions of metaphase chromosomes of selected *Daucus* accessions. **a** *D. carota* subsp. *carota* [PI 478369]; **b** subsp. *carota* [PI 274297]; **c** subsp. *sativus* ('Dolanka'); **d** subsp. *capillifolius* [Ames 30198]; **e** subsp. *gummifer* [PI 478883]; **f** *D. aureus* [PI 319403]; **g** *D. muricatus* [PI 295863]; **h** *D. pumilus*; **i** *D. sahariensis* [Ames 29097]. Scale bar = 5 μm

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Authors' contributions

Conceptualization: DK and EG; Methodology: DK and EG; Formal analysis: DK; Investigation: DK; Resources: DK and EG; Writing – original draft: DK; Writing – review & editing: EG and DK; Visualization: DK; Supervision: EG; Project administration: DK and EG; Founding acquisition: DK and EG. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

The use of all plant materials in this study comply with relevant institutional, national, and international guidelines and legislation. Seeds of all wild accessions were provided by the USDA-ARS North Central Regional Plant Introduction Station (Ames, Iowa, USA) and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK; Gatersleben, Germany), whereas the seeds of cultivated carrot were obtained either from the collections of the Department of Plant Biology and Biotechnology, University of Agriculture in Krakow (Krakow, Poland) or purchased from commercial sources.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Combining genome size and pollen morphology data to study species relationships in the genus *Daucus* (Apiaceae)

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Abstract

Background: The genus *Daucus* (Apiaceae) comprises about 40 wild species and the cultivated carrot, a crop of great economic and nutritional importance. The rich genetic diversity of wild *Daucus* species makes them a valuable gene pool for carrot improvement breeding programs. Therefore, it is essential to have good knowledge of the genome structure and relationships among wild *Daucus* species. To broaden such knowledge, in this research, the nuclear DNA content for 14 *Daucus* accessions and four closely related species was estimated by flow cytometry and their pollen morphology was analyzed by light and scanning electron microscopy (SEM).

Results: The flow cytometric analysis showed a 3.2-fold variation in the mean 2C values among *Daucus* taxa, ranging from 0.999 (*D. carota* subsp. *sativus*) to 3.228 pg (*D. littoralis*). Among the outgroup species, the mean 2C values were 1.775–2.882 pg. The pollen grains of *Daucus* were tricolporate, mainly prolate or perprolate (rarely) in shape, and mainly medium or small (rarely) in size (21.19–40.38 µm), whereas the outgroup species had tricolporate, perprolate-shaped, and medium-sized (26.01–49.86 µm) pollen grains. In the studied taxa, SEM analysis revealed that exine ornamentation was striate, rugulate, perforate, or the ornamentation pattern was mixed. At the time of shedding, all pollen grains were three-celled, as evidenced by DAPI staining. We also found high positive correlations between the length of the polar axis (P) and the length of the equatorial diameter (E) of pollen grains, as well as between P and P/E. However, when comparing cytogenetic information with palynological data, no significant correlations were observed.

Conclusions: This study complements the information on the nuclear DNA content in *Daucus* and provides comprehensive knowledge of the pollen morphology of its taxa. These findings may be important in elucidating the taxonomic relationships among *Daucus* species and can help in the correct identification of gene bank accessions. In a broader view, they could also be meaningful for the interpretation of evolutionary trends in the genus.

Keywords: Crop wild relatives, Flow cytometry, Nuclear DNA content, Palynology, Plant systematics, Plant taxonomy

Background

The genus *Daucus* L. is a member of Apiaceae, a large, complex, and cosmopolitan family of approximately 466 genera and 3820 species that are especially diverse in temperate regions of Eurasia and North America [1]. Although the Apiaceae family is well defined morphologically by a wide range of distinctive characteristics, allowing its constituent taxa to be unambiguously assigned to the family, taxonomic divisions within the family have been extensively discussed [2]. The cultivated carrot (*D. carota* L. subsp. *sativus* Hoffm.) is economically and nutritionally the most significant member of the genus, providing a major source of vitamin A precursors (α- and β-carotene) in the human diet [3]. Based on a

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morpho-anatomical study by Sáenz Laín [4], *Daucus* has traditionally comprised 20 species and has been divided into five sections: *Daucus* L., *Anisactis* DC., *Platyspermum* DC., *Chrysodaucus* Thell, and *Meoides* Lange. This classification was further extended by Rubatzky et al. [5], who listed 25 species. Recently, a number of molecular analyses involving plastid genes (*rbcL*, *matK*), plastid introns (*rpl16*, *rps16*, *rpoC1*), ribosomal internal transcribed spacer (ITS) sequences, chloroplast and mitochondrial DNA restriction sites, nuclear orthologs, and nuclear single nucleotide polymorphisms (SNPs) have been performed to clarify the phylogenetic relationships among *Daucus* species and their close relatives in the subfamily Apioideae [6–19]. These studies have resulted in the division of the genus into two subclades: *Daucus* I and *Daucus* II. *Daucus* I comprises *D. carota* subsp. *carota* L. (the wild ancestor of the cultivated carrot) with all *D. carota* subspecies, as well as several *Daucus* species of Mediterranean origin and some species traditionally placed in other genera, whereas *Daucus* II includes the remaining members of the genus. Following a recent taxonomic revision of *Daucus* by Banasiak et al. [16], the genus has been enlarged to include another 18 species from 9 other genera and now contains about 40 species.

The rich genetic diversity of wild species of *Daucus* could be utilized in carrot breeding programs; hence, it is crucial to better understand the genome structure and relationships among these species. Genome size, defined as the amount of DNA in the holoploid genome of an organism [20], is a fundamental biological characteristic, which can provide insight into the evolutionary background of a species. Knowledge of genome size is important in elucidating the taxonomic relationships among species and tracing evolutionary changes. It may also help resolve conflicting hypotheses concerning the origin of polyploids and serve as additional quality control for species identification in germplasm collections. It can also be useful in a variety of other scientific disciplines, including systematics, phytogeography, phylogeny, and genome sequencing projects, since their scale and cost depend on this parameter [21–25].

For the estimation of nuclear DNA content, flow cytometry has become the predominant method of choice, as it is fast, accurate, and relatively inexpensive [26]. According to the Plant DNA C-value database [27], nuclear DNA amounts have been estimated for 57 members of Apiaceae using flow cytometry; however, it should be noted that this database does not include all estimates.

Cultivated carrot has a relatively small genome of approximately 473 Mb per haploid genome [28, 29]. In the genus *Daucus*, nuclear DNA content estimates by flow cytometry have been reported for several wild species and subspecies, as well as cultivated carrots, revealing great variation in the 2C DNA amount (0.847–3.019 pg) [30–33].

Pollen of each plant species can be described by a variety of characteristics, including size, shape, aperture number and features, and exine ornamentation; thus, the study of pollen morphology is especially important for plant identification and taxonomic research, and can help to determine the relationships among taxa at various taxonomic levels. Palynological characteristics also play a significant role in fields such as phylogeny, paleobotany, archeology, and criminology [34–37]. Although, according to PalDat [38] (the largest database for palynological data), many members of different genera in the family Apiaceae have been subjected to palynological investigation, a comprehensive study on pollen of the genus *Daucus* is still lacking.

Since the systematics of *Daucus* remains under debate, revisions with the use of additional data are necessary to better understand the relationships within *Daucus* species. Therefore, the aims of this research were (1) to estimate the nuclear DNA content in 13 *Daucus* taxa (14 accessions) and four closely related non-*Daucus* species, frequently used in previous phylogenetic and cytotaxonomic studies of *Daucus* [15, 17, 18, 39], by flow cytometry; (2) to investigate the pollen morphology of these taxa by light and scanning electron microscopy (SEM); (3) to determine their pollen nucleus status; (4) to evaluate the taxonomic value of these cytogenomic and palynological data; and (5) to explore whether any correlations exist between genome size and pollen features.

Results

Nuclear DNA content

The 2C values for *Daucus* taxa ranged from 0.999 (*D. carota* subsp. *sativus* [DH]) to 3.228 pg (*D. littoralis*), giving an overall variation of about 3.2-fold (Table 1, Fig. 1). Among the outgroup species, the 2C values varied from 1.775 (*C. platycarpos*) to 2.882 pg (*T. arvensis*). In both groups, the accessions differed in nuclear DNA content ($p < 0.001$).

The taxa belonging to the *Daucus* I subclade exhibited lower genome size compared to the taxa from the *Daucus* II subclade, except for *D. muricatus* (*Daucus* I), whose genome size was much higher than the other accessions of this subclade and even higher than some species of *Daucus* II subclade (Table 1). The monoploid genome size (1C*x*) of all *Daucus* accessions with 18 chromosomes was similar (about 0.5 pg); however, for the species with higher chromosome number it varied from 0.510 to 1.614 pg and did not relate to *n*. Also, in *Daucus* taxa there was no correlation between the nuclear DNA content and chromosome number ($r = 0.521$, $p = 0.06$).

Table 1 Nuclear DNA content of *Daucus* taxa and outgroup species

Taxon	2*n* [a]	Ploidy level	Nuclear DNA content			
			N [b]	2C value (pg, mean ± SE)	2C value range (pg)	1C*x* (pg)
***Daucus* I subclade**						
D. aureus	22	2*x*	21	1.020 ± 0.005 j	0.981–1.059	0.510
D. carota subsp. *capillifolius*	18	2*x*	20	1.058 ± 0.004 i	1.028–1.089	0.529
D. carota subsp. *sativus* (DH)	18	2*x*	8	0.999 ± 0.002 j	0.994–1.011	0.500
D. carota subsp. *sativus* (Dol)	18	2*x*	15	1.003 ± 0.004 j	0.978–1.026	0.501
D. muricatus	22	2*x*	14	2.126 ± 0.005 d	2.103–2.162	1.063
D. rouyi	20	2*x*	16	1.129 ± 0.007 h	1.097–1.178	0.565
D. sahariensis	18	2*x*	10	1.058 ± 0.009 i	1.019–1.129	0.529
D. syrticus	18	2*x*	4	1.038 ± 0.013 ij	1.010–1.070	0.519
***Daucus* II subclade**						
D. conchitae	22	2*x*	14	2.078 ± 0.004 e	2.037–2.095	1.039
D. glochidiatus	44	4*x*	16	2.803 ± 0.011 b	2.758–2.902	0.701
D. guttatus	20	2*x*	14	2.365 ± 0.006 c	2.323–2.410	1.183
D. involucratus	22	2*x*	11	1.953 ± 0.004 f	1.933–1.976	0.976
D. littoralis	20	2*x*	19	3.228 ± 0.009 a	3.150–3.318	1.614
D. pusillus	22	2*x*	22	1.346 ± 0.003 g	1.325–1.378	0.673
Outgroups						
Caucalis platycarpos	20	2*x*	16	1.775 ± 0.003 d	1.761–1.800	0.888
Orlaya daucoides	16	2*x*	23	2.194 ± 0.004 c	2.159–2.244	1.097
O. daucorlaya	14	2*x*	12	2.625 ± 0.005 b	2.589–2.655	1.313
Torilis arvensis	12	2*x*	19	2.882 ± 0.006 a	2.838–2.933	1.441

[a] The 2*n* chromosome numbers were taken from [39]

[b] *N*; number of plants

Means in columns with the same letter were not significantly different at $p < 0.001$. A one-way ANOVA and Tukey's HSD test were conducted separately for *Daucus* taxa and the outgroup species.

Pollen viability and palynological characteristics

The means of pollen viability between *Daucus* taxa differed significantly ($p < 0.001$; Table 2). Despite these differences, the pollen viability rate was relatively high (above 70%), except for *D. pusillus*, whose pollen exhibited lower viability. Similarly, pollen of outgroup species expressed high viability after Alexander's staining, and no significant differences were found between compared accessions.

The means of the pollen morphometric characteristics (the lengths of the polar and equatorial axes) differed significantly ($p < 0.001$; Table 2) within both *Daucus* taxa and the outgroup species. Following the nomenclature for pollen size by Halbritter et al. [36], which classifies pollen as very small (< 10 µm), small (10–25 µm), medium (26–50 µm), large (51–100 µm), or very large (> 100 µm), our observations revealed that the vast majority of *Daucus* taxa (~ 79%) had medium-sized pollen grains; only three taxa (~ 21%) had small pollen grains. The mean length of the polar axis (P) varied from 21.19 (*D. glochidiatus*) to 40.38 µm (*D. aureus*). In all outgroup species, the pollen grains were classified as medium in size, of which the pollen of *Orlaya daucorlaya* showed the highest P parameter (49.86 µm). The average equatorial diameter ranged from 13.20 to 20.21 µm in *Daucus* taxa and from 12.06 to 23.54 µm in the outgroup species. Based on the mean ratio of the polar axis to equatorial diameter (P/E), the pollen grains of *Daucus* taxa were classified as prolate (P/E = 1.33–2.00), except for *D. aureus* and *D. rouyi*, whose pollen were perprolate (P/E > 2.00) in shape, whereas all the outgroup species had perprolate-shaped pollen.

Means in columns with the same letter were not significantly different at $p < 0.001$. A one-way ANOVA and Tukey's HSD test were conducted separately for *Daucus* taxa and the outgroup species.

The SEM analysis performed on pollen samples of 11 taxa (12 accessions) revealed that the pollen grains were tricolporate with narrow colpori (Fig. 2). Considering the pollen outline in the polar view, the examined taxa had triangular pollen grains (see Fig. 2b, m–n, q). The exine ornamentation was striate (elongated ornamentation

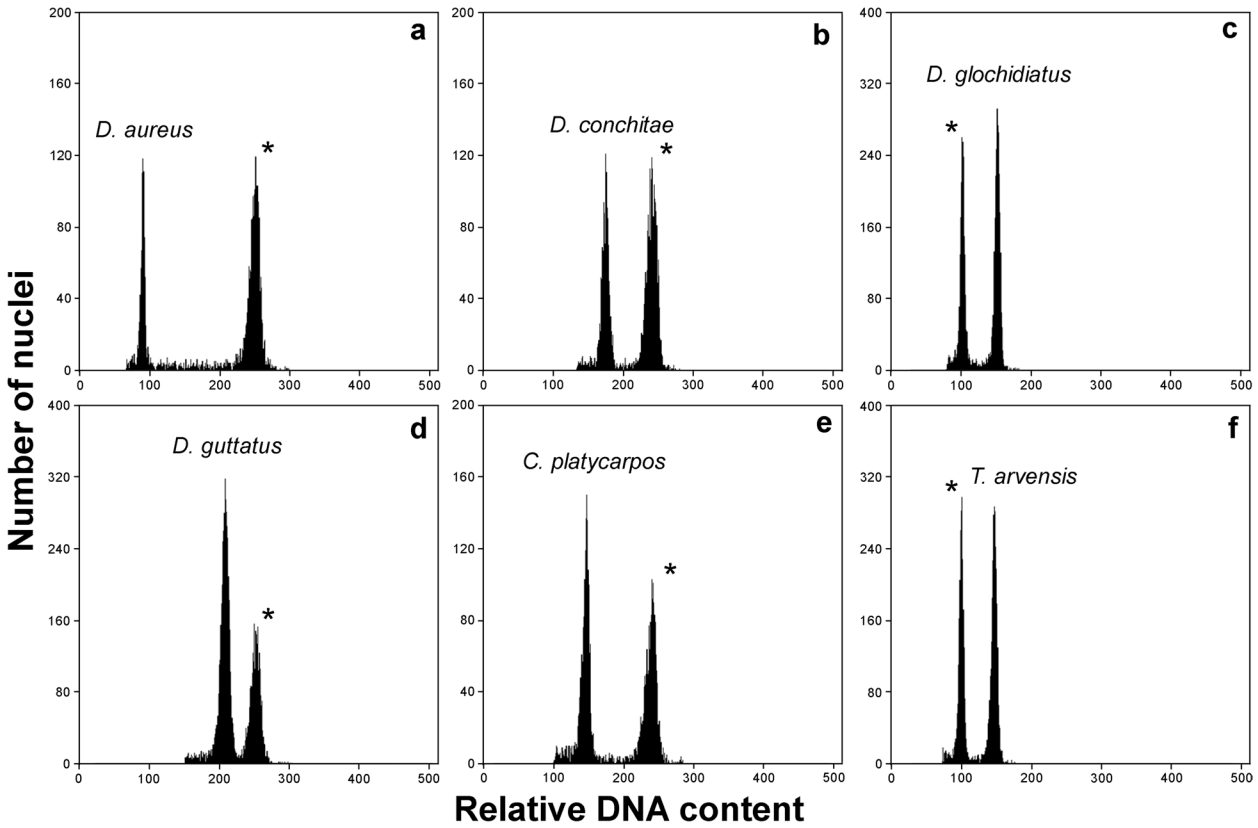

Fig. 1 Exemplary histograms of the relative nuclear DNA content of (**a**) *Daucus aureus*, (**b**) *D. conchitae*, (**c**) *D. glochidiatus*, (**d**) *D. guttatus*, (**e**) *Caucalis platycarpos*, and (**f**) *Torilis arvensis*. Asterisks indicate the internal standards: (**a–b**, **d–e**) *Petunia hybrida* 'P × Pc6' (2C = 2.85 pg), (**c**, **f**) *Solanum lycopersicum* 'Stupicke' (2C = 1.96 pg)

elements separated by parallelly arranged grooves) in *D. conchitae* (Fig. 2b) and *D. rouyi* (Fig. 2p); rugulate (elongated and irregularly arranged ornamentation elements) in *D. carota* subsp. *capillifolius* (Fig. 2d) and *D. involucratus* (Fig. 2l); perforate in *D. guttatus* (Fig. 2j), *O. daucoides* (Fig. 2t), and *O. daucorlaya* (Fig. 2v), or the ornamentation pattern was mixed, *i.e.*, striate-perforate in *D. carota* subsp. *sativus* ('Dolanka') (Fig. 2h), *D. littoralis* (Fig. 2n), and *D. sahariensis* (Fig. 2r); striate-rugulate in *D. carota* subsp. *sativus* (DH) (Fig. 2f); or rugulate-perforate in *T. arvensis* (Fig. 2x).

As evidenced by DAPI staining, at the time of shedding, the pollen of the 14 investigated taxa was three-celled, with a large, weakly stained vegetative nucleus and two smaller, strongly stained sperm cells (Fig. 3). The vegetative nucleus was diffused and more or less round, whereas the sperm cells, depending on the stage of pollen grain development, were round or spindle-shaped and usually located near each other. Although DAPI is a DNA-specific dye, the staining clearly showed the bone-shaped or elliptical outline of the pollen grains, with the apertural areas often visible.

Interspecific relationships within the genus *Daucus*

The UPGMA similarity dendrogram, based on three quantitative parameters (2C DNA content, P, E), divided 14 *Daucus* taxa into three major clusters at a Euclidean distance of 6.5, with a cophenetic correlation of 0.86 (Fig. 4). The first cluster contained three taxa with the largest pollen grains but clearly different 2C DNA content (the variation was about 3.2-fold). In the second cluster, two taxa with the smallest pollen grains and an ~ 2.1-fold variation in 2C value were grouped together. The third cluster was subdivided into two subclusters, one of which included the 18-chromosome taxa form *Daucus* I subclade, with very similar pollen size and 2C values, and two taxa from *Daucus* II subclade with an evidently higher 2C value; the second subcluster comprised two taxa with larger pollen grains and a ~ 1.8-fold variation in 2C DNA content.

According to the Pearson's correlation analysis, high positive correlations were found between the length of the polar axis (P) and the length of equatorial diameter (E) of pollen grains ($r = 0.834$, $p < 0.001$), as well as between P and P/E ($r = 0.823$, $p < 0.001$) (Fig. 5). No

Table 2 Palynological characteristics and pollen viability of *Daucus* taxa and outgroup species

Taxon	Pollen morphology					Pollen viability	
	P [a] (µm, mean ± SE)	E [b] (µm, mean ± SE)	P/E	Shape class [c]	Size class [d]	N [e]	%, mean ± SE
***Daucus* I subclade**							
D. aureus	40.38 ± 0.08 a	18.14 ± 0.04 c	2.23	Perprolate	Medium	1658	93.6 ± 1.8 a
D. carota subsp. *capillifolius*	27.97 ± 0.08 fg	16.69 ± 0.05 d	1.68	Prolate	Medium	1834	89.6 ± 4.6 ab
D. carota subsp. *sativus* (DH)	26.42 ± 0.06 h	15.46 ± 0.05 f	1.71	Prolate	Medium	2145	75.0 ± 2.2 ab
D. carota subsp. *sativus* (Dol)	26.51 ± 0.07 h	14.31 ± 0.04 h	1.85	Prolate	Medium	2080	91.6 ± 1.1 a
D. muricatus	38.59 ± 0.07 b	20.21 ± 0.04 a	1.91	Prolate	Medium	1232	95.5 ± 0.3 a
D. rouyi	33.30 ± 0.04 d	16.23 ± 0.03 e	2.05	Perprolate	Medium	1499	98.6 ± 0.5 a
D. sahariensis	27.73 ± 0.07 g	16.21 ± 0.06 e	1.71	Prolate	Medium	1542	82.7 ± 8.5 ab
D. syrticus	27.58 ± 0.08 g	14.74 ± 0.05 g	1.87	Prolate	Medium	306	98.7
***Daucus* II subclade**							
D. conchitae	31.78 ± 0.08 e	16.89 ± 0.05 d	1.88	Prolate	Medium	1676	87.2 ± 8.2 ab
D. glochidiatus	21.19 ± 0.08 k	15.34 ± 0.06 f	1.38	Prolate	Small	798	93.8 ± 4.3 a
D. guttatus	28.27 ± 0.10 f	16.75 ± 0.05 d	1.69	Prolate	Medium	1528	73.7 ± 5.5 ab
D. involucratus	24.86 ± 0.05 i	13.20 ± 0.04 i	1.88	Prolate	Small	1721	96.1 ± 1.6 a
D. littoralis	37.46 ± 0.10 c	19.26 ± 0.05 b	1.94	Prolate	Medium	1615	96.2 ± 0.8 a
D. pusillus	21.80 ± 0.08 j	14.87 ± 0.05 g	1.47	Prolate	Small	1098	60.9 ± 13.7 b
Outgroups							
Caucalis platycarpos	41.90 ± 0.06 c	20.54 ± 0.03 c	2.04	Perprolate	Medium	1889	78.3 ± 5.5 a
Orlaya daucoides	42.85 ± 0.10 b	20.89 ± 0.06 b	2.05	Perprolate	Medium	1352	79.9 ± 14.0 a
O. daucorlaya	49.86 ± 0.15 a	23.54 ± 0.06 a	2.12	Perprolate	Medium	998	96.3 ± 0.9 a
Torilis arvensis	26.01 ± 0.05 d	12.06 ± 0.02 d	2.16	Perprolate	Medium	1647	90.7 ± 3.3 a

[a] *P*, polar axis

[b] *E*, equatorial diameter

[c] According to the nomenclature of Erdtman [40]

[d] According to the nomenclature of Halbritter et al. [36]

[e] *N*, total number of analyzed pollen grains

significant correlations were observed when comparing cytogenetic information with palynological data.

Discussion

In this study, the nuclear DNA content of 13 taxa (14 accessions) of *Daucus* and four closely related species was estimated by flow cytometry, thus expanding the knowledge of genome size variation in the family Apiaceae. Of these, flow cytometric data for 11 taxa are reported here for the first time. Among the studied taxa, almost all had very small genomes (2C ≤ 2.8 pg), except for *D. littoralis* and *T. arvensis*, whose genome size was categorized as small (2.81–7.00 pg), following the nomenclature of Leitch et al. [41]. It was in agreement with the data included in the Plant DNA C-value database [27], where members of Apiaceae are reported as having mostly very small or small genome sizes; only for a few species genome size exceed 7.00 pg.

To date, the two most extensive cytogenomic studies on the genus *Daucus* have been conducted by Nowicka et al. [32] and Roxo et al. [33]. Nowicka et al. [32] investigated the nuclear DNA content in the collection of diploid members of *Daucus* from different parts of the world, whereas Roxo et al. [33] estimated the 2C values for 16 taxa of the subtribe Daucine from the Macaronesian islands. The results of these works, combined with our findings, revealed an over 3.8-fold variation of the nuclear DNA content within *Daucus*. Some authors suggest that the interspecific variation in DNA content has adaptive significance and correlates with environmental and ecological factors; however, current evidence has been inconclusive so far and does not provide a clear answer as to whether environmental pressure has a relevant impact on plant genome size variation [42–46].

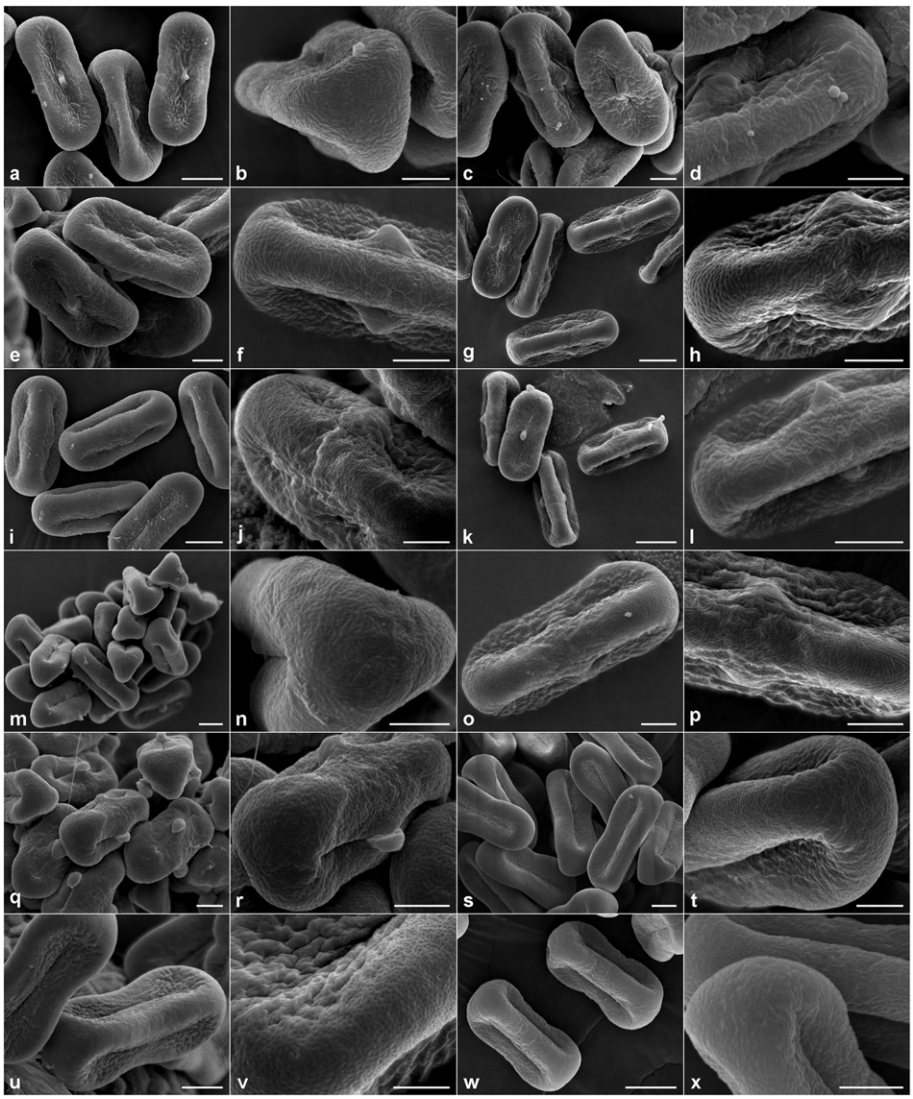

Fig. 2 Pollen morphology and exine ornamentation of selected *Daucus* and related taxa by scanning electron microscopy. (**a–b**) *D. conchitae*; (**c–d**) *D. carota* subsp. *capillifolius*; (**e–f**) *D. carota* subsp. *sativus* (DH); (**g–h**) *D. carota* subsp. *sativus* ('Dolanka'); (**i–j**) *D. guttatus*; (**k–l**) *D. involucratus*; (**m–n**) *D. littoralis*; (**o–p**) *D. rouyi*; (**q–r**) *D. sahariensis*; (**s–t**) *Orlaya daucoides*; (**u–v**) *O. daucorlaya*; (**w–x**) *Torilis arvensis*. Scale bars: 5 µm (**b–f**, **h**, **j**, **l**, **n–r**, **t**, **v**, **x**), 10 µm (**a**, **g**, **i**, **k**, **m**, **s**, **u**, **w**)

In the present research a low variation (less than 6%) in the 2C DNA content of *D. carota* accessions was found. Moreover, all 18-chromosome taxa had similar genome size, which supports the close relationship between these taxa.

Regarding the species for which cytogenomic data have previously been reported, our results are in large part congruent with those obtained by Nowicka et al. [32]. Our estimations for four wild species (*D. involucratus*, *D. littoralis*, *D. muricatus*, and *D. pusillus*) were only slightly higher, with a difference of 4–8%, but such discrepancies may be attributed to the different internal standards that were used by the authors (*Brassica napus* L. 'Bor' and *D. carota* subsp. *sativus* 'Dolanka'). In contrast, the 2C value obtained here for *D. guttatus* conflicted with that of the authors, who found large differences in DNA content for two *D. guttatus* accessions. This could result from their taxonomic misclassification because the germplasm of the *D. guttatus* complex is especially problematic; thus, misidentifications are frequent [47]. Therefore, in this regard, the results are difficult to compare.

The great differences in the 1C*x* value among wild *Daucus* species suggest that in the course of speciation, large-scale chromosomal rearrangements or the accumulation

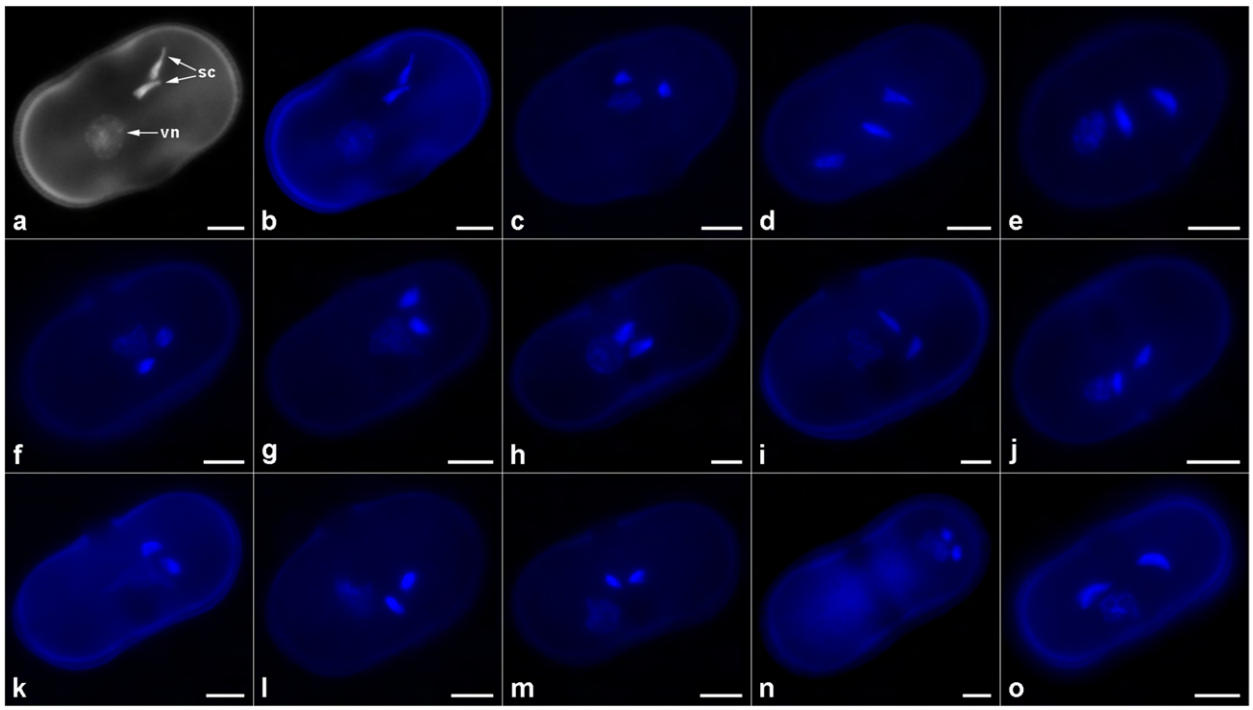

Fig. 3 DAPI-stained pollen grains of selected *Daucus* and related taxa. (**a–b**) *D. conchitae*; (**c**) *D. carota* subsp. *capillifolius*; (**d**) *D. carota* subsp. *sativus* (DH); (**e**) *D. glochidiatus*; (**f**) *D. guttatus*; (**g**) *D. involucratus*; (**h**) *D. littoralis*; (**i**) *D. muricatus*; (**j**) *D. pusillus*; (**k**) *D. rouyi*; (**l**) *D. sahariensis*; (**m**) *D. syrticus*; (**n**) *Orlaya daucoides*; (**o**) *Torilis arvensis*. *sc*, sperm cells; *vn*, vegetative nucleus. Scale bar = 5 μm

of non-coding repetitive DNA sequences (particularly retrotransposons) occurred in the genus.

In this study, the pollen morphology of 17 taxa (18 accessions) of the family Apiaceae (13 *Daucus* and 4 non-*Daucus* taxa) was determined, of which 13 taxa (11 *Daucus* and 2 non-*Daucus*) were examined for the first time. In all taxa, tricolporate and prolate–perprolate-shaped pollen were observed, which is a common feature of the pollen grains of Apiaceae [48–52].

Regarding the exine ornamentation observed by SEM, pollen of the examined *Daucus* taxa was striate, rugulate, perforate, or had a mixed ornamentation pattern; thus, this palynological characteristic may be considered useful for species delimitation within *Daucus*. Exine ornamentation plays an important role in plant systematics; it can be useful for distinguishing closely related genera or sometimes species in the same genus [53, 54]. We also compared the data included in PalDat for species of different genera belonging to the family Apiaceae that were also analyzed by SEM (50 species representing 31 genera) and verified that the pollen grains of most of these species are rugulate and perforate (or only rugulate/perforate), suggesting strong homogeneity for this trait in Apiaceae. Nonetheless, outside PalDat, some other types of exine ornamentation in Apiaceae have also been reported in the literature, *e.g.*, cerebroid, pertectate, and verrucate [50–52, 55].

At the time of shedding, the pollen grains of the studied taxa were three-celled, as revealed by DAPI staining, which is a characteristic feature of pollen in the Apiaceae family [56]. In nature, most flowering plants produce pollen that is arrested at the two-celled stage, containing one vegetative cell and one generative cell, and only around 30% of species shed three-celled pollen (one vegetative cell, two sperm cells) at anthesis [57, 58]. Compared to two-celled pollen, three-celled pollen grains are inherently short-lived [59], and they are also more hydrated [60].

Many examples of correlations between genome size and phenotypic traits at the nuclear, cellular, and tissue levels can be found in the literature. Studies have shown that the amount of DNA is associated with nuclear and cell volume, cell size, cell cycle duration, stomatal cell size, cell density [61–65], seed mass [66], leaf mass per unit area [67], and flowering time [68].

Although the nuclear DNA content did not correlate with the pollen features of *Daucus* taxa, this parameter can be of great use in distinguishing individual taxa within some groups of taxa with a similar pollen size (Fig. 4), *e.g.*, taxa with small ($P < 25$ μm) pollen grains

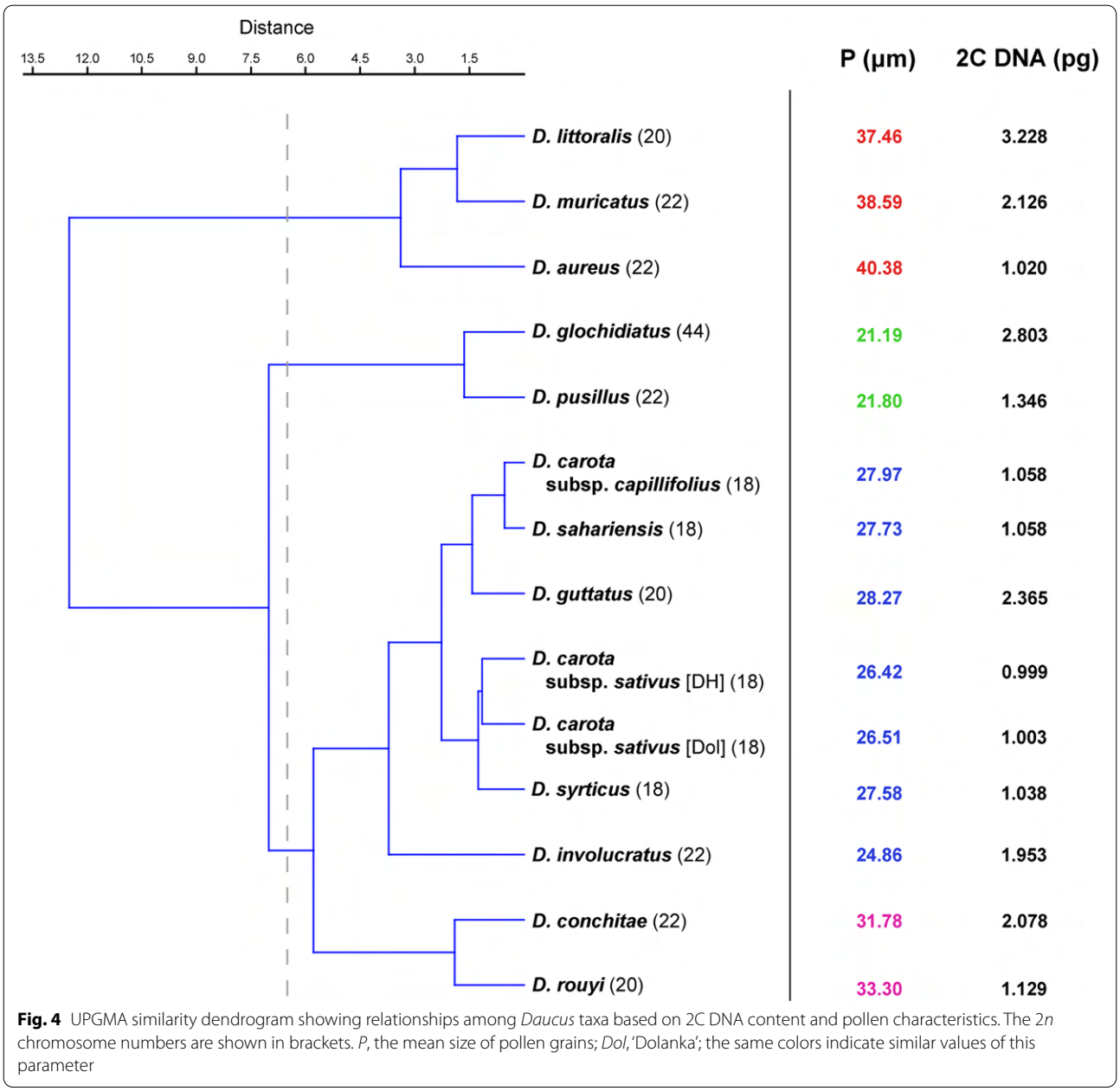

Fig. 4 UPGMA similarity dendrogram showing relationships among *Daucus* taxa based on 2C DNA content and pollen characteristics. The 2*n* chromosome numbers are shown in brackets. *P*, the mean size of pollen grains; *Dol*,'Dolanka'; the same colors indicate similar values of this parameter

(*D. glochidiatus*, *D. involucratus*, and *D. pusillus*) differed in terms of 2C DNA content; the same relation was observed in taxa with P ≈ 30–34 µm (*D. conchitae* and *D. rouyi*), as well as in those with the largest pollen grains, *i.e.*, P ≈ 37–41 µm (*D. aureus*, *D. muricatus*, and *D. littoralis*). However, in the group of taxa with pollen grains of P ≈ 26–29 µm, only *D. guttatus* can be separated from the others based on 2C DNA content. In the case of whole plant morphology, for example, two morphologically similar species, *D. conchitae* (2C = 2.08 pg) and *D. guttatus* (2C = 2.37 pg), can be easily distinguished based on their DNA content. On the other hand, some taxa that shared very similar DNA content and pollen size, *e.g.*, *D. carota* subsp. *capillifolius* and carrot, were morphologically distinct.

Wild *Daucus* species may play an essential role in carrot breeding programs, as they could be a valuable potential source of agronomically important genes. Thus, to effectively utilize this germplasm, it is crucial to determine the species boundaries and relationships within *Daucus* [47, 69]. Considering that the correct identification of species is a prerequisite for further use, the

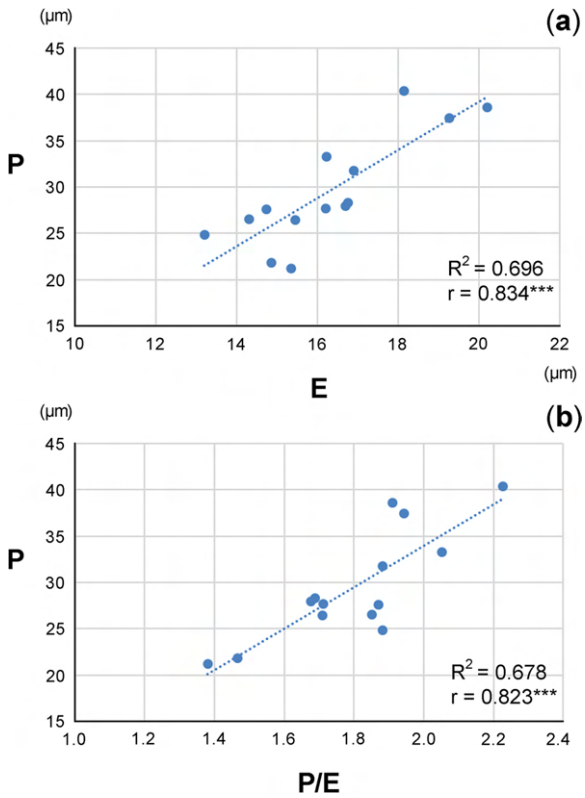

Fig. 5 Relationship between (**a**) polar axis (P) and equatorial diameter (E) of pollen grains and (**b**) P and P/E ratio among *Daucus*. Three asterisks (***) indicate statistical significance at $p < 0.001$; *r*, Pearson's correlation coefficient; R^2, coefficient of determination

application of supplementary methods for this purpose is essential. Therefore, the assessment of relative nuclear DNA content by flow cytometry can be a good choice for simple, rapid, and low-cost screening of genebank accessions during their identification and maintenance, even at seedling stage [24, 70, 71], which could be further combined with palynological measurements to help reliably identify species, as evidenced in this study.

Conclusions

The present study significantly complements the available information on the nuclear DNA content in the genus *Daucus* and provides comprehensive knowledge of the pollen morphology of its taxa. These results may be of great importance in elucidating the taxonomic relationships among *Daucus* species and can help in the correct identification of gene bank accessions. From a broader view, the findings of this work could also be meaningful for the interpretation of the evolutionary trends in the genus. Nonetheless, to better understand the relationships within *Daucus* in the phylogenetic context, further studies comprising the remaining taxa from the genus, as well as the taxa from different genera that have recently been included in *Daucus*, are needed.

Methods

Plant material

A total of 18 accessions representing 17 taxa (species or subspecies) were examined in this study, including 12 accessions of wild *Daucus* taxa, a reference doubled haploid carrot line, one carrot cultivar, and four accessions of closely related non-*Daucus* species (Table 3). Seeds of all wild accessions were provided by the USDA-ARS North Central Regional Plant Introduction Station (Ames, Iowa, USA), whereas carrot seeds were either purchased commercially or obtained from the collections of the Department of Plant Biology and Biotechnology, University of Agriculture in Krakow (Krakow, Poland).

The seeds were germinated in soil-filled pots and grown in a growth chamber at 18 °C with a long-day photoperiod of 16/8 h (light/dark) for the first few weeks, then transferred to greenhouse conditions (26/14 °C ± 2 °C, day/night temperature; long-day photoperiod) until flowering. In the case of two cultivated carrot accessions, the plants were first vernalized in a cold chamber at 5 °C for three months, then returned to the greenhouse for flowering.

Flow cytometric measurements of nuclear DNA content

For nuclear DNA content measurements, 8–23 plants per accession, depending on availability, were used. Young leaves were collected from plants grown in a growth chamber and samples for flow cytometric analysis were prepared as previously described [72] using—for nuclei isolation—Galbraith's buffer [73], supplemented with 1% (w/v) polyvinylpyrrolidone (PVP-10, MW 10,000; Sigma-Aldrich, St. Louis, USA), ribonuclease A (RNase A, 50 μg mL^{-1}; Sigma-Aldrich), and propidium iodide (PI, 50 μg mL^{-1}; Sigma-Aldrich). *Solanum lycopersicum* L. 'Stupicke' (2C = 1.96 pg; [74]) were used as an internal standard for *D. glochidiatus*, *D. littoralis*, *O. daucorlaya*, and *T. arvensis*, while for the remaining accessions, *Petunia hybrida* Vilm. 'P × Pc6' (2C = 2.85 pg; [75]) was applied. The nuclei suspension was analyzed using a CyFlow SL Green flow cytometer (Partec GmbH, Münster, Germany) equipped with a high-grade solid-state laser ($\lambda_{em} = 532$ nm), long-pass filter RG 590 E, DM 560 A, and side and forward scatters. The PI fluorescence was measured in 3000–5000 nuclei per sample. For histogram evaluation, FloMax software (Partec GmbH, Münster, Germany) was applied. The coefficient of variation (CV) of the G_0/G_1 peak of sample species ranged between 2.68 and 5.96%. Nuclear DNA content was calculated using the linear

Table 3 List of *Daucus* and closely related non-*Daucus* accessions used in this study

Taxon [a]	Seed source [b]/Accession no. [c]	Country of origin
***Daucus* I subclade**		
D. aureus	USDA/PI 319403	Israel
D. carota subsp. *capillifolius*	USDA/PI 279764	Libya
D. carota subsp. *sativus* (DH)	RZ/DH1	The Netherlands
D. carota subsp. *sativus* (Dol)	Commercial/'Dolanka'	Poland
D. muricatus	USDA/PI 295863	Spain
D. rouyi	USDA/PI 674284	Tunisia
D. sahariensis	USDA/Ames 29096	Tunisia
D. syrticus	USDA/Ames 29108	Tunisia
***Daucus* II subclade**		
D. conchitae	USDA/Ames 25835	Turkey
D. glochidiatus	USDA/PI 285038	Australia
D. guttatus	USDA/PI 652233	Iran
D. involucratus	USDA/PI 652332	Greece
D. littoralis	USDA/PI 295857	Israel
D. pusillus	USDA/PI 349267	Uruguay
Outgroups		
Caucalis platycarpos	USDA/PI 649446	Germany
Orlaya daucoides	USDA/PI 649477	Turkey
O. daucorlaya	USDA/PI 649478	Greece
Torilis arvensis	USDA/PI 649391	Syria

[a] The taxonomic classification is according to [15, 16]

[b] *RZ*, Rijk Zwaan vegetable breeding company, Lier, the Netherlands; *USDA*, USDA-ARS North Central Regional Plant Introduction Station (NCRPIS), Ames, Iowa, USA

[c] *Ames*, Ames numbers are assigned to carrots and other Apiaceae maintained in the NCRPIS; *PI*, USDA Plant Introduction numbers are permanent numbers assigned to germplasm accessions in the National Plant Germplasm System (NPGS); *DH1*, a doubled haploid orange Nantes-type carrot

relationship between the ratio of the 2C peak positions sample/standard on a histogram of fluorescence intensities.

Pollen viability, morphology, and nucleus status

Pollen viability was assessed using Alexander's staining method [76]. Fresh pollen was collected from fully open flowers (from the anthers after dehiscence) of the greenhouse-grown plants onto microscope slides (samples were taken from 1–3 randomly chosen inflorescences per individual, 3–5 plants per accession; except for *D. syrticus* and *D. glochidiatus*, for which only one and two plants, respectively, were available), then a drop of Alexander's stain was applied to each slide and covered with a cover glass. The slides were examined under an Axio Imager. M2 microscope (Carl Zeiss, Göttingen, Germany), and the number of viable (dark red cytoplasm with a green wall) and non-viable (entirely green) pollen grains were counted, with a minimum of 300 pollen grains per slide. The pollen viability was expressed as a percentage of viable pollen.

Pollen size was determined using samples of Alexander-stained pollen that had been used for the viability test. The polar axis (P) and equatorial diameter (E) of the pollen grains were measured from microphotographs captured with a Canon PowerShot G10 digital camera (Canon, Tokyo, Japan) attached to the same microscope as above. At least 100 viable pollen grains per plant (3–5 plants per accession; except for *D. syrticus* and *D. glochidiatus*, for which only one and two plants, respectively, were available) were measured. The terminology for pollen size follows that of Halbritter et al. [36]. The pollen of each accession was classified into a shape class based on the ratio of the polar axis to the equatorial diameter (P/E), according to the nomenclature proposed by Erdtman [40].

For SEM analysis, pollen samples from the fully open flowers of 12 accessions were collected into gelatin capsules and stored in an exsiccator until use. Dry pollen grains were mounted on stubs and sputter-coated with gold using a JFC-1100E ion sputter coater (JEOL, Tokyo, Japan). The palynological characteristics (exine ornamentation and aperture number) were examined under a JSM-5410 scanning electron microscope with a wolfram cathode (JEOL, Tokyo, Japan). The terminology for exine ornamentation follows that of Halbritter et al. [36].

To determine the pollen nucleus status (expressed as the number of pollen nuclei in pollen grains after anther dehiscence), pollen samples of 14 taxa (12 *Daucus* taxa and two outgroup species) were collected in the same way as for the viability test, then mounted in a drop of 4′,6-diamidino-2-phenylindole (DAPI) solution (2.5 μg^{-1} DAPI, 7.7 mM Tris–HCl, 10 mM spermine tetrahydrochloride, 10 mM NaCl, 2.2% hexylene glycol, and 0.25% Triton™ X-100; mixed in a 1:1 ratio with glycerol), and covered with a cover glass. The slides were examined under the same microscope using the fluorescence mode and an appropriate filter set for DAPI (Zeiss filter set 02: $\lambda_{ex} = 365$ nm, $\lambda_{em} > 420$ nm). The microphotographs were captured using a BV MV camera (Applied Spectral Imaging, Edingen-Neckarhausen, Germany).

Statistical analyses

For quantitative parameters, means and standard errors of the means were calculated for each accession and subjected to a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test at a significance level of at least $p = 0.05$ using Statistica v. 13.3 (TIBCO Software Inc., USA). For nuclear DNA content estimation and pollen morphology and viability, the mean of measurements/counts for one plant was considered a single replication. Statistical analyses were conducted separately for the *Daucus* taxa and the outgroup species.

To determine the relationships among the *Daucus* taxa, an unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis with Euclidean distance was performed based on nuclear DNA content and pollen morphology (P, E) data using Past v. 3.22 software [77].

To identify relationships among cytogenetic (2C DNA content, somatic chromosome number) and palynological (P, E, P/E) data within the genus *Daucus*, Pearson's correlation analysis was carried out using Statistical.

Abbreviations

CWRs: Crop wild relatives; DAPI: 4′,6-Diamidino-2-phenylindole; E: Equatorial diameter; P: Polar axis; SEM: Scanning electron microscopy; UPGMA: Unweighted pair-group method with arithmetic mean.

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Authors' contributions

Conceptualization: DK, EG; Methodology: DK, ES; Formal analysis: DK, ES; Investigation: DK, ES; Resources: DK, ES, EG; Writing—original draft: DK; Writing—review & editing: DK, ES, EG; Visualization: DK; Supervision: EG; Project administration: DK, EG; Founding acquisition: DK, EG. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

The use of all plant materials in this study complies with relevant institutional, national, and international guidelines and legislation. Seeds of all wild accessions were provided by the USDA-ARS North Central Regional Plant Introduction Station (Ames, Iowa, USA), whereas the seeds of cultivated carrots were obtained either from the collections of the Department of Plant Biology and Biotechnology, University of Agriculture in Krakow (Krakow, Poland) or purchased from commercial sources.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Comparative Fruit Morphology and Anatomy of Wild Relatives of Carrot (*Daucus*, Apiaceae)

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Abstract: Fruit morphological and anatomical characteristics are essential in the taxonomy of Apiaceae. *Daucus* L. is one of the most important genera of the family Apiaceae, as it contains the cultivated carrot, a crop of great economic importance, and about 40 wild species that could serve as potential sources of genetic diversity for crop improvement. However, the taxonomic and phylogenetic relationships among these species have not yet been fully clarified. In this study, we comparatively investigated the fruit morphology and anatomy of 13 *Daucus* taxa and four closely related non-*Daucus* species using light and scanning electron microscopy to evaluate the taxonomic value of these characteristics. A wide range of variations was observed in the fruit morpho-anatomical characteristics across the taxa and revealed several diagnostically valuable features, thus proving to be taxonomically useful. For *Daucus*, the observed differences included the fruit size (2.1–8.4 mm), shape (from ellipsoid to oblong), and weight (0.079–1.349 g/100 fruits), as well as the fruit surface sculpturing and some anatomical characteristics, i.e., the presence/absence and size of vittae, the shape and size of vascular bundles, and the shape of exocarp cells. This study contributes to a better understanding of the relationships among the genus *Daucus*.

Keywords: Apioideae; carpology; crop wild relatives; Daucinae; mericarp; plant systematics; schizocarp; Torilidinae

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1. Introduction

The genus *Daucus* L. belongs to the large and complex family Apiaceae, which comprises approximately 3820 species in 466 genera that are widely distributed all around the world, especially in the temperate regions of Eurasia and North America [1]. This cosmopolitan family is considered one of the most economically important families, and it includes a number of food crops, herbs, and spices [2]. *Daucus* contains carrot (*D. carota* subsp. *sativus* Hoffm.), the only cultivated member of the genus, which is a crop of great importance for human nutrition as it serves as a major source of α- and β-carotene (vitamin A precursors) in the diet [3,4]. The taxonomic and phylogenetic relationships among *Daucus* species have not yet been fully clarified. Traditionally, the genus comprised 20–25 species, as inferred from morphological and anatomical data [2,5]. However, recent studies based on different molecular data have led to a better understanding of the species boundaries and phylogenetic relationships among *Daucus* and its close relatives in the Apioideae subfamily [6–13]. Following these revisions, the genus has been extended to include nine other genera, and it now contains about 40 species positioned in two main clades [11].

The wild species of *Daucus* are widespread in the temperate parts of the Northern Hemisphere, most commonly in the Mediterranean region, which is considered the center of this genus's diversity; however, few species occur in South America, Australia, and tropical Africa [14,15]. They are mostly herbaceous biennials, rarely annuals [14], but a few rosette treelets (endemic to Macaronesia) also exist [16]. Most *Daucus* species are

diploids with chromosome numbers of 2*n* = 16, 18, 20, or 22; however, some tetra- and hexaploids have also been reported [15,17,18]. Regarding the genome size within the genus, nuclear DNA content estimates based on flow cytometry are available for several wild species and subspecies, as well as for many cultivated carrots, ranging from 0.920 to 3.228 pg/2C DNA [19–21].

The fruit of Apiaceae is typically a schizocarp that splits at maturity into two—usually equal—ribbed, one-seeded mericarps. Each mericarp has five primary ribs: three dorsal (one median and two lateral) and two marginal (closest to the commissure), which are separated by furrows (valleculae). The primary ribs enclose one or more vascular bundles that are often associated with schizogenous secretory canals (rib oil ducts). Another set of secretory canals (vittae) are located in the valleculae and the commissural area but are not associated with the vasculature. In some groups, secondary ribs develop from the valleculae, and they have no vascular bundles (see Figure 1) [1,22,23]. The fruit's structural characteristics, especially the number and distribution of vittae and vascular bundles, as well as rib/wing morphology, have proven to be exceptionally useful for the taxonomy of Apiaceae (e.g., [24–32]). Regarding *Daucus*, several decades ago, Sáenz Laín [5] published a taxonomic monograph of the genus based on morpho-anatomical analyses, providing some observations of the fruit morphology and anatomy of *Daucus* taxa; however, this was a largely intuitive classification that did not contain specimen citations, detailed descriptions, or distribution maps [33]. More recently, Mezghani et al. [34] studied the patterns of phenotypic diversity of fruits among Tunisian *Daucus* germplasm collection, whereas Wojewódzka et al. [35] investigated fruit evolution in many members of the Apiaceae tribe Scandiceae, including some *Daucus* taxa, and outgroups to assess adaptive shifts associated with the evolutionary switches between anemochory and epizoochory, as well as to identify possible dispersal syndromes.

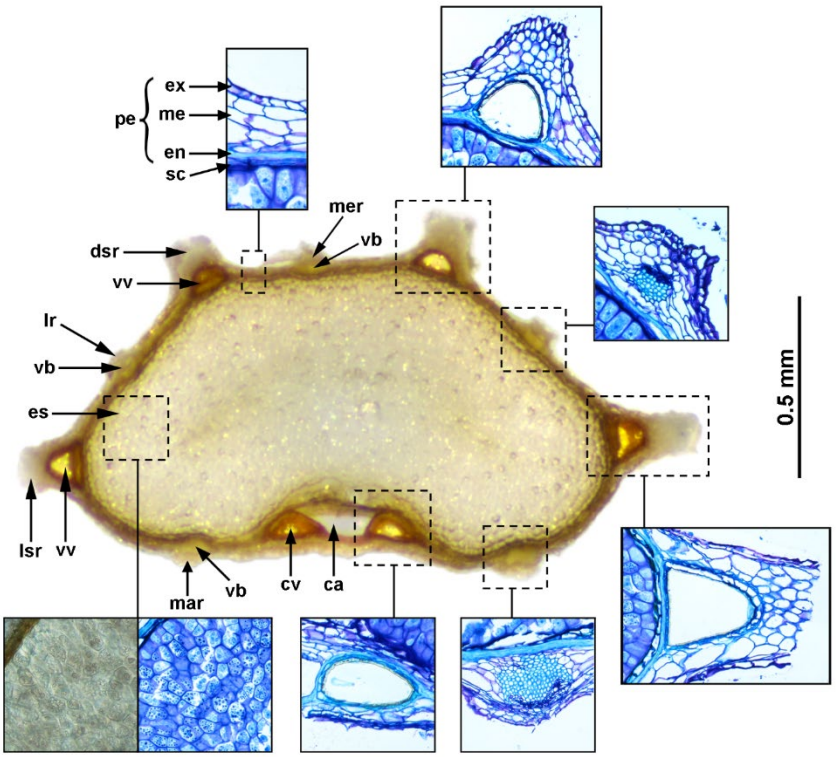

Figure 1. Transverse section of a mericarp of *Daucus* sp., indicating the anatomical structures considered in this study and their terminology. The insets show the corresponding structures, as seen by light microscopy. Abbreviations: ca, cavity; cv, commissural vitta; dsr, dorsal secondary rib; en, endocarp; es, endosperm; ex, exocarp; lr, lateral primary rib; lsr, lateral secondary rib; mar, marginal primary rib; me, mesocarp; mer, median primary rib; pe, pericarp; sc, seed coat; vb, vascular bundle; vv, vallecular vitta.

To address the rising need for food and to ensure food security for a constantly growing population, plant breeders require access to new genetic resources that could be used in crop breeding programs to expand the genetic variation of crops that has been lost during domestication. Such a large pool of genetic diversity can be found in crop wild relatives, which—due to their high adaptability to a wide range of habitats and environmental conditions—represent an important reservoir of agronomically important genes [36–38]. In this context, wild *Daucus* species may play a crucial role in the process of improving modern agriculture, being a valuable source of genes potentially useful for breeding purposes, e.g., for producing new crop varieties that could be disease-resistant, tolerant to abiotic stress, higher-yielding, male-sterile, or more nutritious [9,14]. In light of this, a better understanding of species relationships within the genus *Daucus* may greatly contribute to the development of future carrot breeding programs.

Given the significance of wild *Daucus* species and the great economic importance of the cultivated carrot, as well as the taxonomical usefulness of fruit structural characteristics in Apiaceae, this study aimed to compare the fruit morphology and anatomy of *Daucus* taxa using light and scanning electron microscopy (SEM) and to evaluate the diagnostic value of these characteristics. In this study, which is a continuation of our previous work [21], we selected a representative sample that covered the two main *Daucus* subclades (13 taxa) and four closely related non-*Daucus* species. We used the same accessions that have commonly been used in previous phylogenetic and (cyto)taxonomic research on the genus [9,12,13,21,39].

2. Materials and Methods

2.1. Plant Material

The study materials were ripe fruits (mericarps) of 13 *Daucus* taxa (14 accessions) and four closely related non-*Daucus* species (outgroup). The *Daucus* accessions comprised 12 wild taxa belonging to *Daucus* subclades I and II, as well as two cultivated carrots. The fruit samples of wild *Daucus* and non-*Daucus* accessions were provided by the USDA-ARS North Central Regional Plant Introduction Station (Ames, IA, USA), whereas the fruits of the carrot accessions were either purchased from commercial sources or obtained from the collections of the Department of Plant Biology and Biotechnology, University of Agriculture in Krakow (Krakow, Poland). The following taxa were used (chromosome numbers [17,18] and accession numbers [PI = USDA Plant Introduction numbers] are given in brackets): *Daucus aureus* Desf. (2*n* = 22; PI 319403), *D. conchitae* Greuter (2*n* = 22; Ames 25835), *D. carota* subsp. *capillifolius* (Gilli) C. Arbizu (2*n* = 18; PI 279764), *D. carota* subsp. *sativus* Hoffm. (2*n* = 18; DH1, a doubled haploid orange Nantes-type carrot), *D. carota* subsp. *sativus* (2*n* = 18; 'Dolanka'), *D. glochidiatus* (Labill.) Fisch & C.A. Mey (2*n* = 44; PI 285038), *D. guttatus* Sm. (2*n* = 20; PI 652233), *D. involucratus* Sm. (2*n* = 22; PI 652332), *D. littoralis* Sm. (2*n* = 20; PI 295857), *D. muricatus* (L.) L. (2*n* = 22; PI 295863), *D. pusillus* Michx. (2*n* = 22; PI 349267), *D. rouyi* Spalik & Reduron (2*n* = 20; PI 674284), *D. sahariensis* Murb. (2*n* = 18; Ames 29096), *D. syrticus* Murb. (2*n* = 18; Ames 29108), *Caucalis platycarpos* L. (2*n* = 20; PI 649446), *Orlaya daucoides* (L.) Greuter (2*n* = 16; PI 649477), *O. daucorlaya* Murb. (2*n* = 14; PI 649478), and *Torilis arvensis* (Huds.) Link (2*n* = 12; PI 649391).

2.2. Fruit Morphology

To characterize fruit morphology, 50 dry mericarps of each accession were placed on graph paper and photographed with a Flexacam C1 digital camera (Leica Microsystems, Heerbrugg, Switzerland) under a Leica S6D stereomicroscope (Leica Microsystems). The images were processed using Leica Application Suite X (Leica Microsystems) software, and the mericarp length (L) and width (W) were measured using AxioVision 4.8.2 software (Carl Zeiss MicroImaging, Jena, Germany). The fruit shape was described on the basis of the mean ratio of the mericarp length to width (L/W), and the following shape

classes were used: ovoid (L/W ≤ 1.5), ellipsoid (L/W = 1.6–2.0), and narrowly ellipsoid or oblong (L/W ≥ 2.0), according to Lee et al. [40] and Mustafina et al. [41].

For scanning electron microscopy (SEM) analysis, dry fruit samples were mounted on stubs and sputter-coated with gold using a JFC-1100E ion sputter coater (JEOL, Tokyo, Japan); then, the dorsal side of the mericarps was examined under a JSM-5410 scanning electron microscope with a wolfram cathode (JEOL). The terminology used to describe the fruit surface sculpturing pattern was adopted from Stearn [42] and Ostroumova [43].

The fruit weight of each accession was expressed as grams per 100 mericarps and estimated by weighing four subsamples (each containing 50 randomly selected mericarps) using a WPS 510/C analytical balance (Radwag, Radom, Poland). The mean value was then calculated to obtain the weight of 100 mericarps.

2.3. Fruit Anatomy

For anatomical examination, 5–10 fruit samples (schizocarps or individual mericarps) of each accession were rehydrated in distilled water for 24–48 h, fixed in freshly prepared FAA (formalin, glacial acetic acid, and 70% ethanol, 6:4:90, *v*/*v*/*v*) for 48–72 h at room temperature, and stored in 70% ethanol at 4°C until further use. The samples were then dehydrated in a graded ethanol series (80% and 90% for 2 h each) and left overnight in absolute ethanol. The dehydrated material was embedded in Technovit® 7100 resin (Kulzer, Hanau, Germany), following the manufacturer's protocol, with minor modifications involving prolonged infiltration with embedding solutions, i.e., the material was treated with increasing concentrations of Technovit relative to ethanol (1:3, 1:1, 3:1, *v*/*v*) for 24 h each and then left in pure Technovit for 5 days. The fixation, dehydration, and infiltration steps were performed on an orbital shaker (150 rpm) at room temperature, with 15 min vacuum pumping during each solution change. When polymerized, cross-sections of 4–8 μm thickness were made using a Leica RM2145 rotary microtome (Leica Microsystems, Wetzlar, Germany) with a Leica TC-65 carbide blade (Leica Microsystems). The sections were then stained with 0.2% (*w*/*v*) toluidine blue O (Sigma-Aldrich, Steinheim, Germany) for 30–60 s, mounted in Entellan® (Merck, Darmstadt, Germany), and analyzed under an Axio Imager.M2 microscope (Carl Zeiss, Göttingen, Germany). Three quantitative anatomical characteristics were measured (from five mericarps per accession): width of commissural vittae, width of vallecular vittae, and pericarp thickness. The terminology used to describe fruit anatomy follows that of Kljuykov et al. [22,23] and Wojewódzka et al. [35].

Another fruit sample was rehydrated in distilled water for 24 h and hand-sectioned using a disposable razor blade. The sections were then photographed under a stereomicroscope with the same camera as described in Section 2.2.

The transverse section of an exemplary mericarp showing the anatomical structures considered in this study, along with their terminology, is given in Figure 1.

2.4. Statistical Analysis

For each accession, the means and standard errors (SE) of the means were calculated for the measured quantitative parameters and then subjected to a one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). The differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Fruit Morphology

The fruits of the studied taxa were schizocarps consisting of two homomorphic mericarps. The mericarps were pale yellow to brown in color and ovoid to oblong in shape in dorsal view (Figure 2 and Table 1). All taxa had spiny fruits, except for *D. rouyi* (Figure 2l), whose fruits were winged; however, since the material was mostly obtained from gene bank collections, the fruits often had broken spikes/wings or were devoid of these structures. In almost all taxa, the primary ribs were more or less inconspicuous or rarely prominent, covered with hairs or pointed thorns, whereas the secondary ribs (two dorsal and two lateral) were remarkably prominent (Figure 2). *Torilis arvensis*, however, had numerous additional secondary ribs covering almost the entire surface of the fruit (Figure 2r).

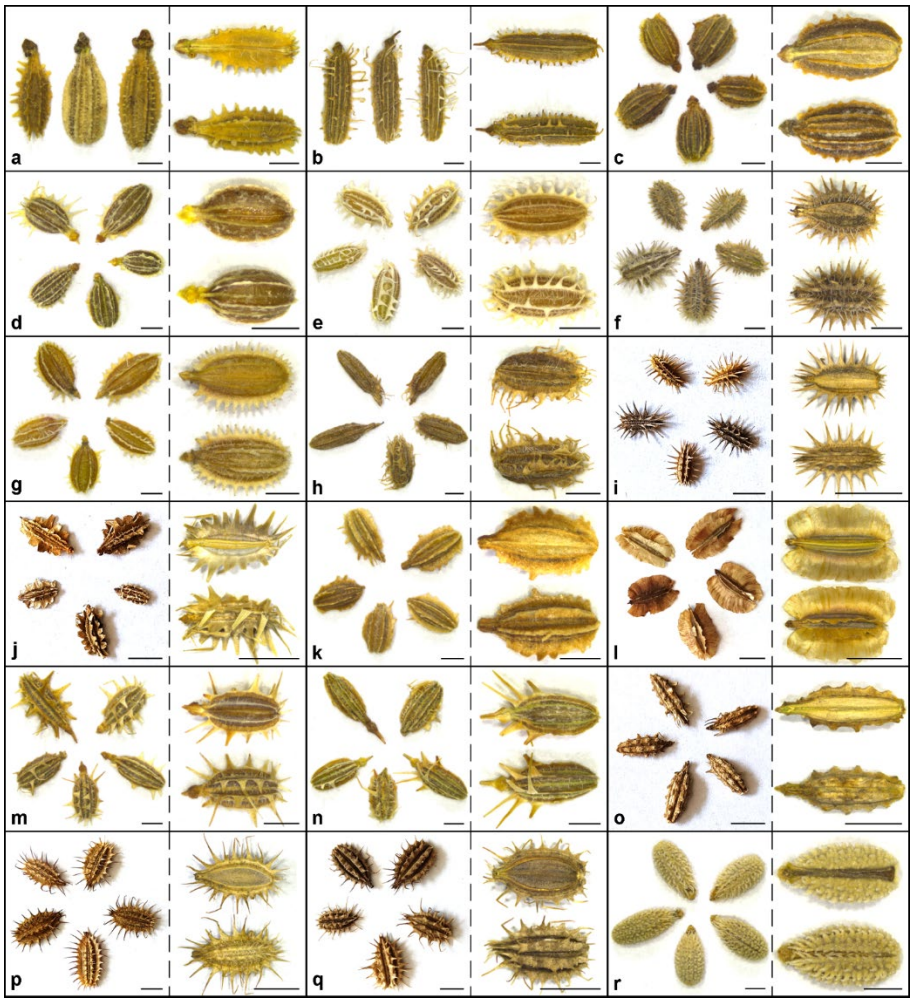

Figure 2. Variation in fruit morphology of the investigated *Daucus* and closely related non-*Daucus* taxa. The insets show magnified views of the dorsal (lower) and ventral (upper) sides of the mericarps. (**a**) *D. aureus*; (**b**) *D. carota* subsp. *capillifolius*; (**c**) subsp. *sativus* (DH); (**d**) subsp. *sativus* ('Dolanka'); (**e**) *D. conchitae*; (**f**) *D. glochidiatus*; (**g**) *D. guttatus*; (**h**) *D. involucratus*; (**i**) *D. littoralis*; (**j**) *D. muricatus*; (**k**) *D. pusillus*; (**l**) *D. rouyi*; (**m**) *D. sahariensis*; (**n**) *D. syrticus*; (**o**) *Caucalis platycarpos*; (**p**) *Orlaya daucoides*; (**q**) *O. daucorlaya*; (**r**) *Torilis arvensis*. Scale bars: 1 mm (**a**–**h**,**k**,**m**,**n**,**r**); 5 mm (**i**,**j**,**l**,**o**–**q**).

Table 1. Fruit (mericarp) morphological characteristics of the investigated *Daucus* and closely related non-*Daucus* (outgroup) taxa.

Taxon	Length (L; mm)		Width (W; mm)		L/W	Shape	100 Fruit Weight (g)
	Min–Max	Mean ± SE	Min–Max	Mean ± SE			Mean ± SE
***Daucus* I subclade**							
D. aureus	2.5–4.4	3.3 ± 0.06 h	1.2–2.1	1.5 ± 0.03 de	2.2	NE	0.136 ± 0.002 f–h
D. carota subsp. *capillifolius*	4.0–6.5	4.9 ± 0.07 g	1.1–1.7	1.4 ± 0.02 d–f	3.6	OB	0.206 ± 0.002 f
D. carota subsp. *sativus* (DH)	2.3–3.4	2.8 ± 0.04 i	1.5–1.7	1.6 ± 0.01 d	1.7	E	0.117 ± 0.003 f–h
D. carota subsp. *sativus* ('Dolanka')	1.6–2.8	2.1 ± 0.04 k	0.9–1.7	1.3 ± 0.02 fg	1.7	E	0.139 ± 0.005 f–h
D. muricatus	4.5–8.4	6.5 ± 0.12 e	2.1–4.0	2.6 ± 0.05 c	2.5	NE	1.076 ± 0.020 d
D. rouyi	6.8–12.1	8.4 ± 0.13 c	5.6–10.9	7.7 ± 0.15 a	1.1	OV	1.349 ± 0.012 c
D. sahariensis	2.0–3.8	2.7 ± 0.05 i	0.9–1.6	1.3 ± 0.03 e–g	2.1	NE	0.098 ± 0.002 gh
D. syrticus	1.9–3.7	2.7 ± 0.06 i	0.9–1.7	1.2 ± 0.03 fg	2.2	NE	0.080 ± 0.002 h
***Daucus* II subclade**							
D. conchitae	1.9–3.7	2.5 ± 0.05 i–k	0.8–1.5	1.1 ± 0.03 fg	2.2	NE	0.106 ± 0.003 gh
D. glochidiatus	1.8–3.0	2.2 ± 0.04 jk	0.9–1.5	1.2 ± 0.02 fg	1.9	E	0.079 ± 0.004 h
D. guttatus	2.2–3.8	2.8 ± 0.05 i	1.1–1.8	1.4 ± 0.03 d–f	2.1	NE	0.109 ± 0.001 f–h
D. involucratus	2.5–3.4	2.9 ± 0.03 i	0.9–1.6	1.1 ± 0.02 g	2.6	OB	0.098 ± 0.002 gh
D. littoralis	4.8–6.7	5.7 ± 0.07 f	2.1–3.3	2.6 ± 0.04 c	2.3	NE	0.596 ± 0.011 e
D. pusillus	2.0–2.9	2.5 ± 0.03 ij	1.1–1.6	1.3 ± 0.02 e–g	2.0	NE	0.091 ± 0.002 gh
Outgroup							
Caucalis platycarpos	5.8–8.2	7.1 ± 0.07 d	2.3–3.5	2.8 ± 0.03 c	2.6	OB	1.664 ± 0.023 b
Orlaya daucoides	8.7–14.0	11.4 ± 0.18 a	4.2–8.1	5.8 ± 0.11 b	2.0	NE	3.407 ± 0.061 a
O. daucorlaya	6.8–12.5	10.1 ± 0.18 b	3.8–7.3	5.8 ± 0.09 b	1.8	E	3.451 ± 0.035 a
Torilis arvensis	2.2–3.6	2.7 ± 0.03 i	1.2–1.8	1.4 ± 0.02 d–g	2.0	NE	0.178 ± 0.005 fg

Means followed by the same letter in a column were not significantly different at $p \leq 0.05$. E, ellipsoid; NE, narrowly ellipsoid; OB, oblong; OV, ovoid; SE, standard error.

The mean mericarp length (L) varied from 2.1 (*D. carota* subsp. *sativus* 'Dolanka') to 11.4 mm (*O. daucoides*), whereas the average mericarp width (W) ranged from 1.1 (*D. conchitae* and *D. involucratus*) to 7.7 mm (*D. rouyi*) (Table 1). The ratio of these two parameters (L/W) was recorded in the range between 1.1 (*D. rouyi*) and 3.6 (*D. carota* subsp. *capillifolius*).

A closer look at the dorsal side of the mericarps, as examined under SEM (Figure 3), showed that the vast majority of *Daucus* taxa exhibited more or less rugose fruit surface sculpturing (Figure 3b–f,h,k,l). The most distinct pattern was found in *D. aureus* (Figure 3a), whose whole fruit surface was densely covered with tubercles (tuberculate type of sculpturing). Moreover, a few other or mixed types were detected. In *D. guttatus*, a rugose–tuberculate pattern was observed, i.e., rugose in the furrows between ribs, tuberculate on the surface of the secondary ribs (Figure 3g). *Daucus littoralis* showed a lineolate–tuberculate (lineolate furrows and tuberculate secondary ribs) surface (Figure 3i), whereas *D. rouyi* displayed ribbed–striate sculpturing (Figure 3j). In *D. muricatus*, the furrows were not clearly seen, but the surface of the secondary ribs was tuberculate (Figure 3m).

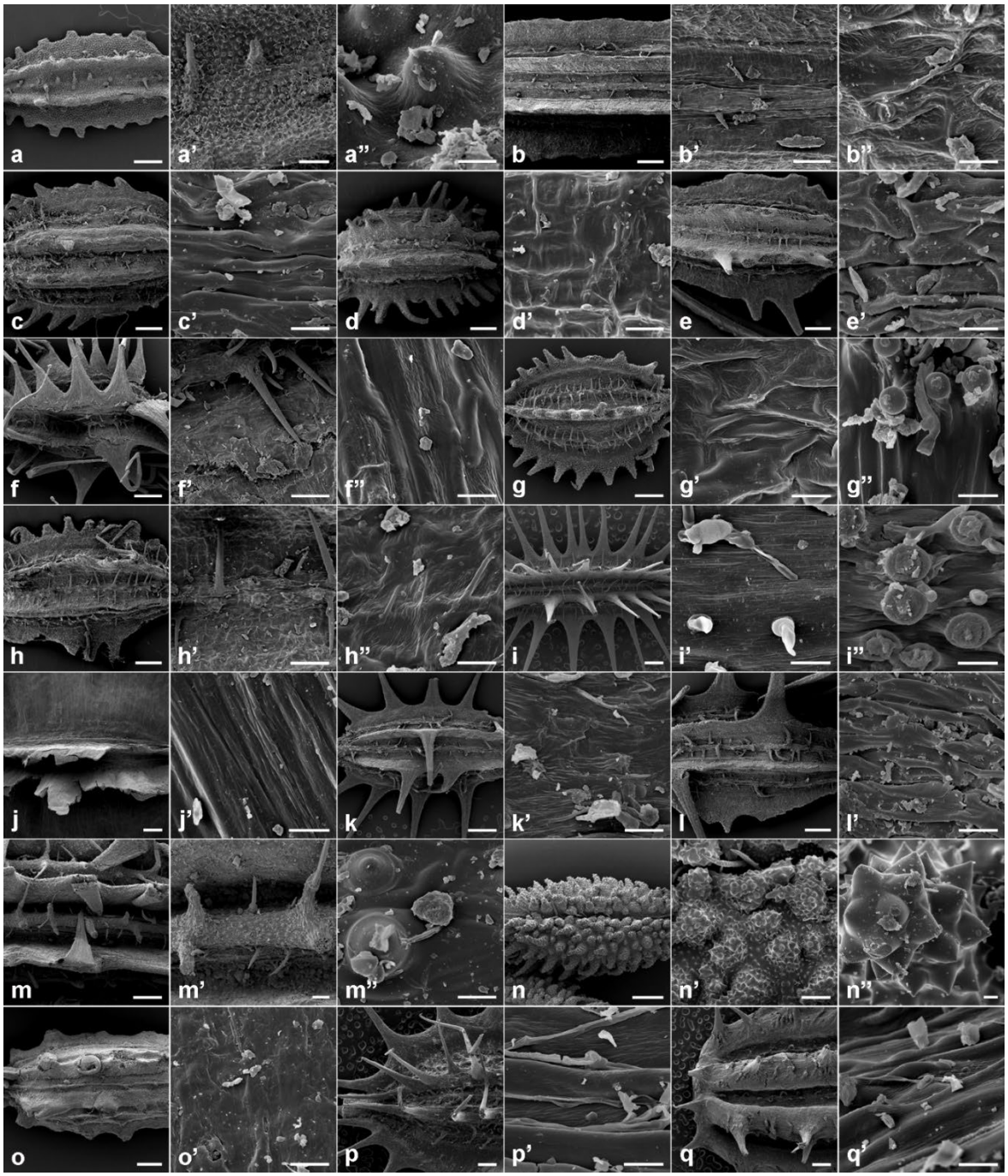

Figure 3. Fruit morphology and its surface micromorphology of the investigated *Daucus* and closely related non-*Daucus* taxa by scanning electron microscopy. (**a**–**a′′**) *D. aureus*: (**a**) dorsal view and close-ups on (**a′**) the median primary rib and (**a′′**) tubercle; (**b**–**b′′**) *D. carota* subsp. *capillifolius*: (**b**) dorsal view and close-ups on (**b′**) the median primary rib and (**b′′**) surface between ribs; (**c**,**c′**) subsp. *sativus* (DH): (**c**) dorsal view, (**c′**) close-up on the surface between ribs; (**d**–**d′**) subsp. *sativus* ('Dolanka'): (**d**) dorsal view, (**d′**) close-up on the surface between ribs; (**e**,**e′**) *D. pusillus*: (**e**) dorsal view, (**e′**) close-up on the surface between ribs; (**f**–**f′′**) *D. conchitae*: (**f**) dorsal view and close-ups on (**f′**) the primary rib and (**f′′**) surface between ribs; (**g**–**g′′**) *D. guttatus*: (g) dorsal view and close-ups on (**g′**) the surface between ribs and (**g′′**) tubercles; (**h**–**h′′**) *D. involucratus*: (**h**) dorsal view and close-ups on (**h′**) the median primary rib and (**h′′**) surface between ribs; (**i**–**i′′**) *D. littoralis*: (**i**) dorsal view and close-ups on (**i′**) the surface between ribs and (**i′′**) tubercles; (**j**,**j′**) *D. rouyi*: (**j**) dorsal view, (**j′**) close-up on the surface of the wing; (**k**,**k′**) *D. sahariensis*: (**k**) dorsal view, (**k′**) close-up on the surface between ribs; (**l**,**l′**) *D. syrticus*: (**l**) dorsal view, (**l′**) close-up on the surface between ribs; (**m**–**m′′**) *D. muricatus*: (**m**) dorsal view and close-ups on (**m′**) the primary rib and (**m′′**) tubercles; (**n**–**n′′**) *Torilis arvensis*: (**n**) dorsal view and close-ups on (**n′**) the additional secondary ribs and (**n′′**) tubercle;

(**o**–**o′**) *Caucalis platycarpos*: (**o**) dorsal view, (**o′**) close-up on the surface between ribs; (**p**,**p′**) *Orlaya daucoides*: (**p**) dorsal view, (**p′**) close-up on the surface between ribs; (**q**,**q′**) *O. daucorlaya*: (**q**) dorsal view, (**q′**) close-up on the surface between ribs. Scale bars: 600 μm (**i**,**j**,**o**–**q**); 400 μm (**a**,**f**,**g**,**k**,**m**,**n**); 300 μm (**b**–**e**,**h**,**l**); 100 μm (**a′**,**b′**,**f′**,**h′**,**m′**,**n′**); 10 μm (**c′**–**e′**,**g′**,**i′**–**l′**,**o′**–**q′**,**a″**,**b″**,**f″**–**i″**,**m″**,**n″**).

Among the outgroup species, variations in the types of sculpturing were also observed. In *T. arvensis*, the secondary ribs were densely covered with pointed tubercles (Figure 3n); *Caucalis platycarpos* exhibited a smooth surface (Figure 3o), while both *O. daucoides* and *O. daucorlaya* showed an undulate sculpturing pattern (Figure 3p,q, respectively).

In all cases, the outlines of the exocarp cells were not visible.

The lowest mean weight of 100 fruits (mericarps) was recorded for *D. glochidiatus* (0.079 g/100 fruits) and *D. syrticus* (0.080 g/100 fruits) (Table 1). *Orlaya daucorlaya* and *O. daucoides* had the heaviest fruits (3.451 and 3.407 g/100 fruits, respectively).

3.2. Fruit Anatomy

The mericarp outline in the transverse section of almost all examined taxa was slightly compressed dorsally (Figures 4–6 and Table 2), except for *C. platycarpos*, which was slightly compressed laterally (Figure 6d).

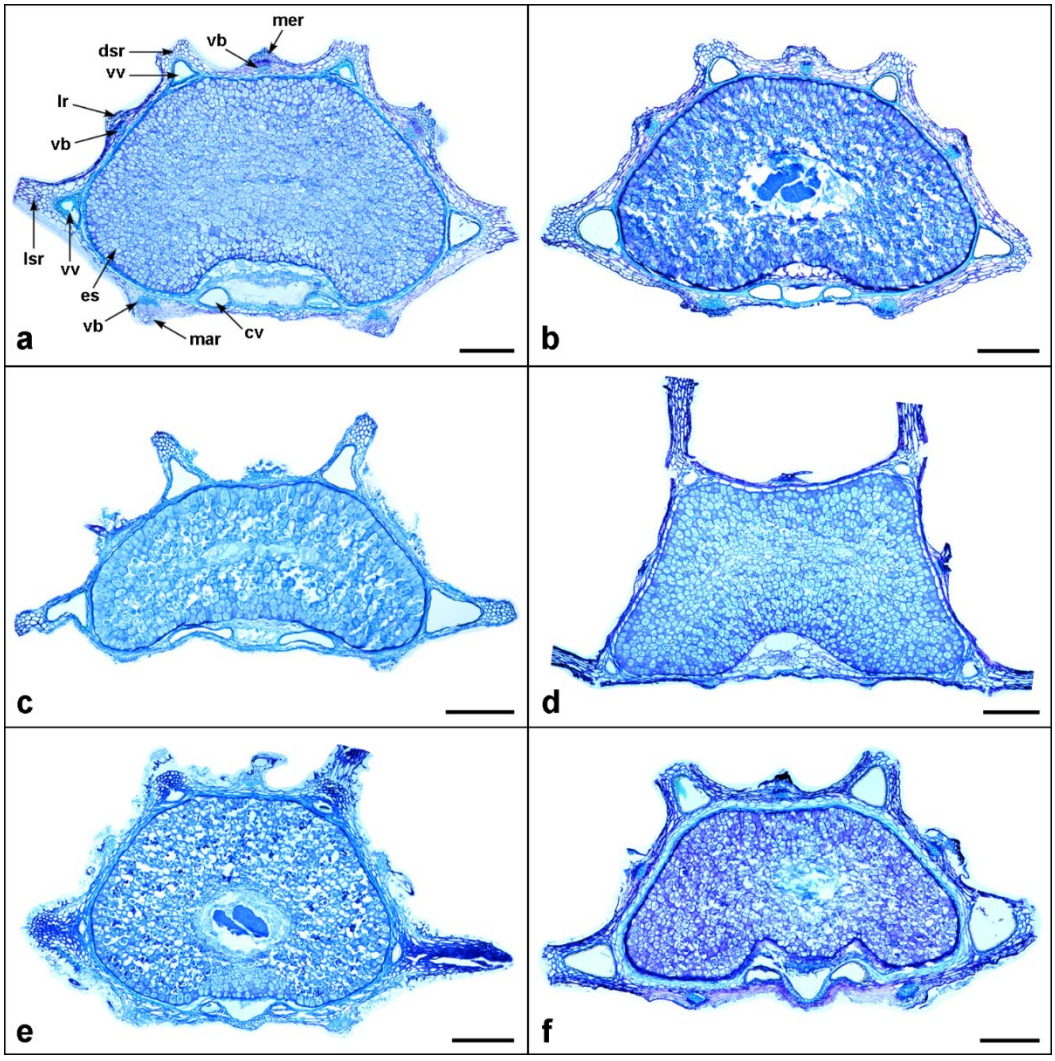

Figure 4. Mericarp structure of the investigated *Daucus* taxa, as seen in a transverse section. (**a**) *D. carota* subsp. *sativus* (DH); (**b**) subsp. *sativus* ('Dolanka'); (**c**) subsp. *capillifolius*; (**d**) *D. conchitae*; (**e**) *D. glochidiatus*; (**f**) *D. guttatus*. Abbreviations: cv, commissural vitta; dsr, dorsal secondary rib; es,

endosperm; lr, lateral primary rib; lsr, lateral secondary rib; mar, marginal primary rib; mer, median primary rib; vb, vascular bundle; vv, vallecular vitta. Scale bar = 200 μm.

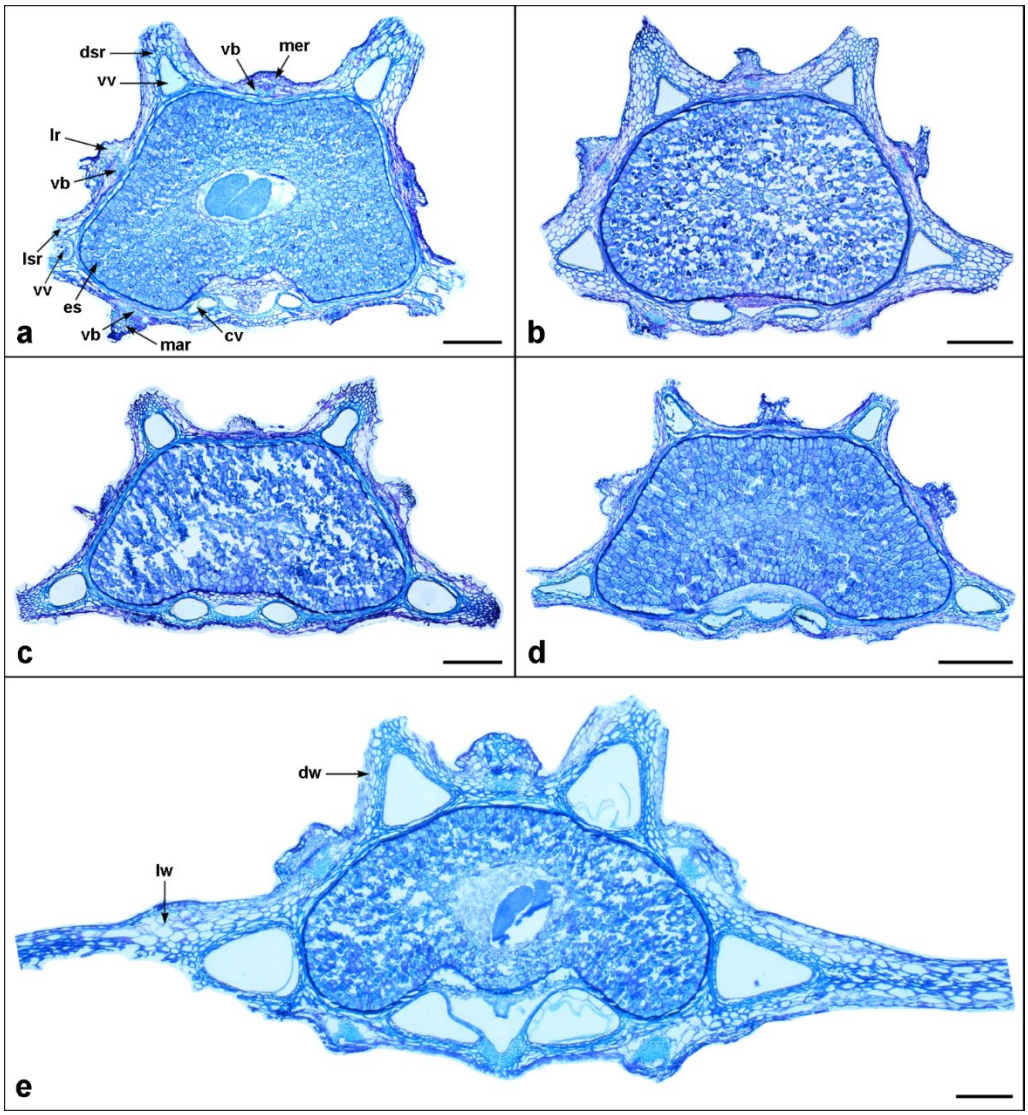

Figure 5. Mericarp structure of the investigated *Daucus* taxa, as seen in a transverse section. (**a**) *D. involucratus*; (**b**) *D. pusillus*; (**c**) *D. sahariensis*; (**d**) *D. syrticus*; (**e**) *D. rouyi*. Abbreviations: cv, commissural vitta; dsr, dorsal secondary rib; dw, dorsal wing; es, endosperm; lr, lateral primary rib; lsr, lateral secondary rib; lw, lateral wing; mar, marginal primary rib; mer, median primary rib; vb, vascular bundle; vv, vallecular vitta. Scale bar = 200 μm.

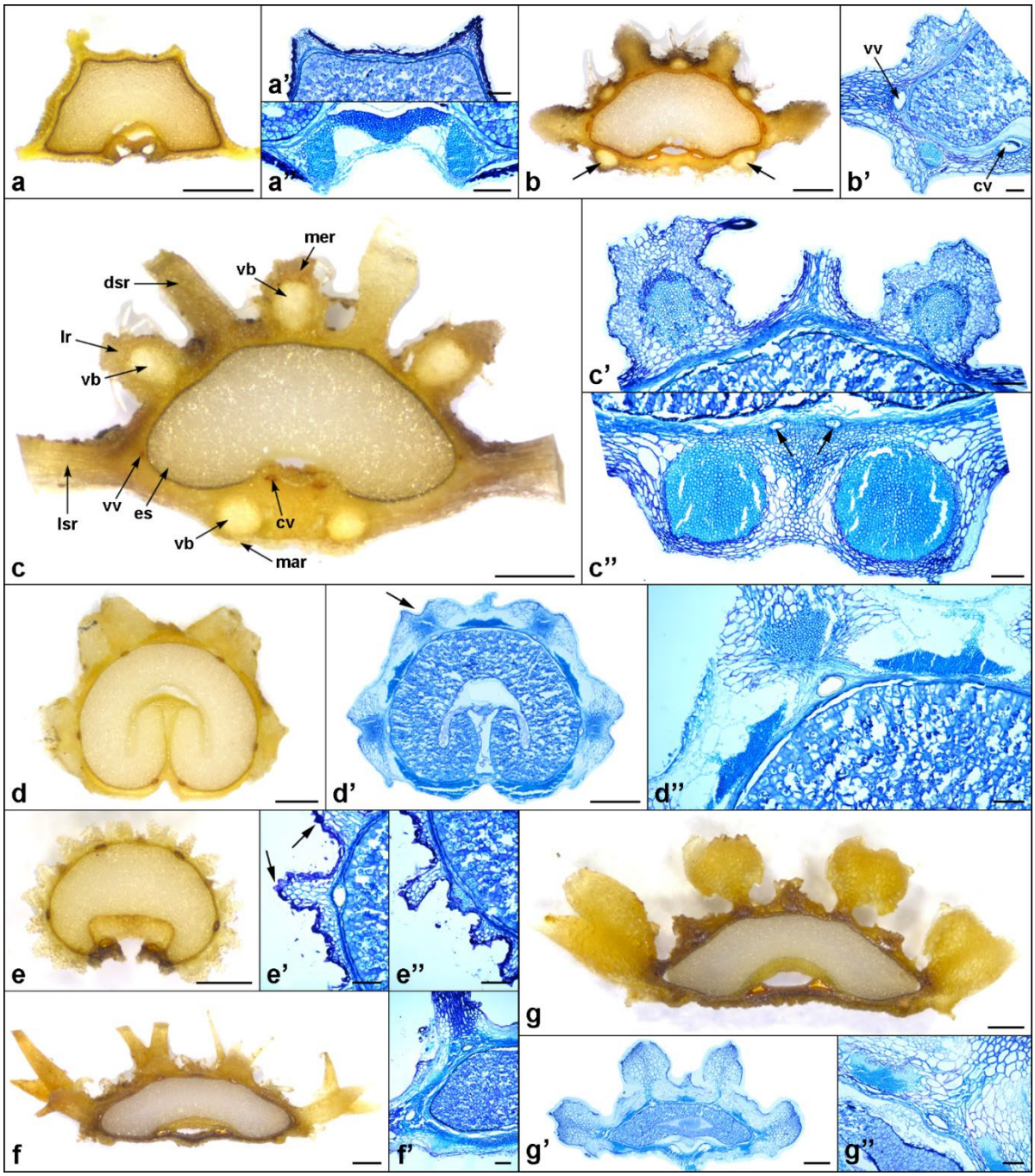

Figure 6. Mericarp structure of the investigated *Daucus* and closely related non-*Daucus* taxa, as seen in a transverse section. (**a**–**a''**) *D. aureus*; (**a'**) the upper part of the mericarp showing the absence of the vallecular vittae; (**a''**) M-shaped vascular bundle at the commissural side; (**b**,**b'**) *D. littoralis*, arrows indicate the larger vascular bundles in the marginal primary ribs; (**c**–**c''**) *D. muricatus*; (**c'**) the upper part of the mericarp showing two primary ribs with vascular bundles and the secondary rib in the middle enclosing the vallecular vitta; (**c''**) vascular bundles in the marginal primary ribs, arrows indicate commissural vittae; (**d**–**d''**) *Caucalis platycarpos*, arrow in (**d'**) indicates the sunken apex of the secondary rib; (**d''**) close-up on the upper part of the mericarp showing the flattened and elongated vascular bundles and a patch of collenchyma above the vallecular vitta; (**e**–**e''**) *Torilis arvensis*; (**e'**) close-up on the secondary rib enclosing the vallecular vitta, arrows indicate tubercles covering the exocarp; (**e''**) close-up on the part of the mericarp with secondary ribs and the primary rib in the middle; (**f**,**f'**) *Orlaya daucoides*; (**g**–**g''**) *O. daucorlaya*; (**g''**) close-up on the upper part of the mericarp showing the vasculature and the vallecular vitta. Abbreviations: cv, commissural vitta; dsr, dorsal secondary rib; es, endosperm; lr, lateral primary rib; lsr, lateral secondary rib; mar,

marginal primary rib; mer, median primary rib; vb, vascular bundle; vv, vallecular vitta. Scale bars: 0.5 mm (**a**–**g**,**d′**,**g′**); 100 μm (**a′**–**c′**,**e′**,**f′**,**a″**,**c″**–**e″**,**g″**).

Table 2. Fruit (mericarp) anatomical characteristics of the investigated *Daucus* and closely related non-*Daucus* (outgroup) taxa.

Taxon	Width (μm)		Pericarp Thickness (μm)	Mericarp Outline [a]	Exocarp [b]	Hypendocarp	Endosperm [c]	Surface Micromorphology
	VV	CV						
***Daucus* I subclade**								
D. aureus	absent	absent	51 ± 5 c–e	SCD	T	–	F/C	Tuberculate
D. carota subsp. *capillifolius*	139 ± 6 bc	181 ± 16 cd	28 ± 1 e	SCD	–	–	F/C	Rugose
D. carota subsp. *sativus* (DH)	91 ± 4 ef	115 ± 6 e–g	38 ± 1 e	SCD	–	–	F/C	Rugose
D. carota subsp. *sativus* ('Dolanka')	82 ± 8 fg	78 ± 3 f–h	32 ± 3 e	SCD	–	–	F/C	Rugose
D. muricatus	33 ± 2 i	83 ± 6 f–h	108 ± 7 ab	SCD	–	–	F/C	Tuberculate
D. rouyi	168 ± 7 a	200 ± 7 c	132 ± 9 a	SCD	–	–	F/C	Ribbed–striate
D. sahariensis	75 ± 4 f–h	122 ± 5 e–g	42 ± 5 e	SCD	A	–	F/C	Rugose
D. syrticus	70 ± 3 f–h	144 ± 14 de	48 ± 4 de	SCD	–	–	F/C	Rugose
***Daucus* II subclade**								
D. conchitae	64 ± 3 gh	71 ± 5 gh	35 ± 2 e	SCD	–	–	F/C	Rugose
D. glochidiatus	53 ± 3 hi	53 ± 3 h	34 ± 6 e	SCD	A	–	F/C	N/A
D. guttatus	87 ± 4 e–g	108 ± 8 e–g	51 ± 4 c–e	SCD	–	–	F/C	Rugose–tuberculate
D. involucratus	75 ± 2 f–h	81 ± 2 f–h	31 ± 2 e	SCD	–	–	F/C	Rugose
D. littoralis	110 ± 5 de	127 ± 8 ef	80 ± 5 bc	SCD	–	–	F/C	Lineolate–tuberculate
D. pusillus	85 ± 2 e–g	125 ± 5 ef	38 ± 5 e	SCD	–	–	F/C	Rugose
Outgroup								
Caucalis platycarpos	93 ± 2 ef	87 ± 3 f–h	117 ± 7 a	SCL	–	–	MG	Smooth
Orlaya daucoides	125 ± 5 cd	273 ± 17 b	129 ± 6 a	SCD	–	+	F/C	Undulate
O. daucorlaya	150 ± 12 ab	329 ± 26 a	118 ± 13 a	SCD	–	+	F/C	Undulate
Torilis arvensis	81 ± 3 fg	106 ± 7 e–g	77 ± 6 cd	SCD	T	–	MG	Tuberculate

[a] Mericarp outline in transverse section. [b] The presence or absence of exocarp protuberances or appendages. [c] Endosperm shape at commissure. Means followed by the same letter in a column were not significantly different at $p \leq 0.05$. A, cells with triangular appendages; CV, width of commissural vittae; F/C, flat or more or less concave; MG, mushroom-like grooved; N/A, not analyzed; SCD, slightly compressed dorsally; SCL, slightly compressed laterally; SE, standard error; T, covered with tubercles; VV, width of vallecular vittae.

Although the primary ribs of *Daucus* fruits were not prominent compared to the secondary ones (Figures 3–6), they were distinctly large in *D. muricatus* but still not larger than the secondary ribs (Figures 3m and 6c). The primary ribs were more or less similar in size and shape, whereas the secondary ribs often differed, with lateral secondary ribs usually longer than dorsal ones. The number of ribs in the mericarps was typically constant among taxa, i.e., five primary and four secondary, except for *D. littoralis*, in which mericarps with one additional primary and one additional secondary rib were rarely found (Figure 7a). Among the outgroup, the rib architectural pattern was similar to *Daucus*, i.e., more or less inconspicuous primary ribs and prominent secondary ribs; however, some distinct features of the latter were observed. The secondary ribs of *C. platycarpos* were wide and thick, often with a sunken apex (Figure 6d). *Orlaya daucoides* sometimes had bifurcated secondary ribs (Figure 6f), whereas those of *O. daucorlaya* were massive and thick, often clavate-shaped, with a thin base (Figure 6g). The fruits of *T. arvensis* were characterized by the presence of numerous additional secondary ribs (Figures 3n and 6e).

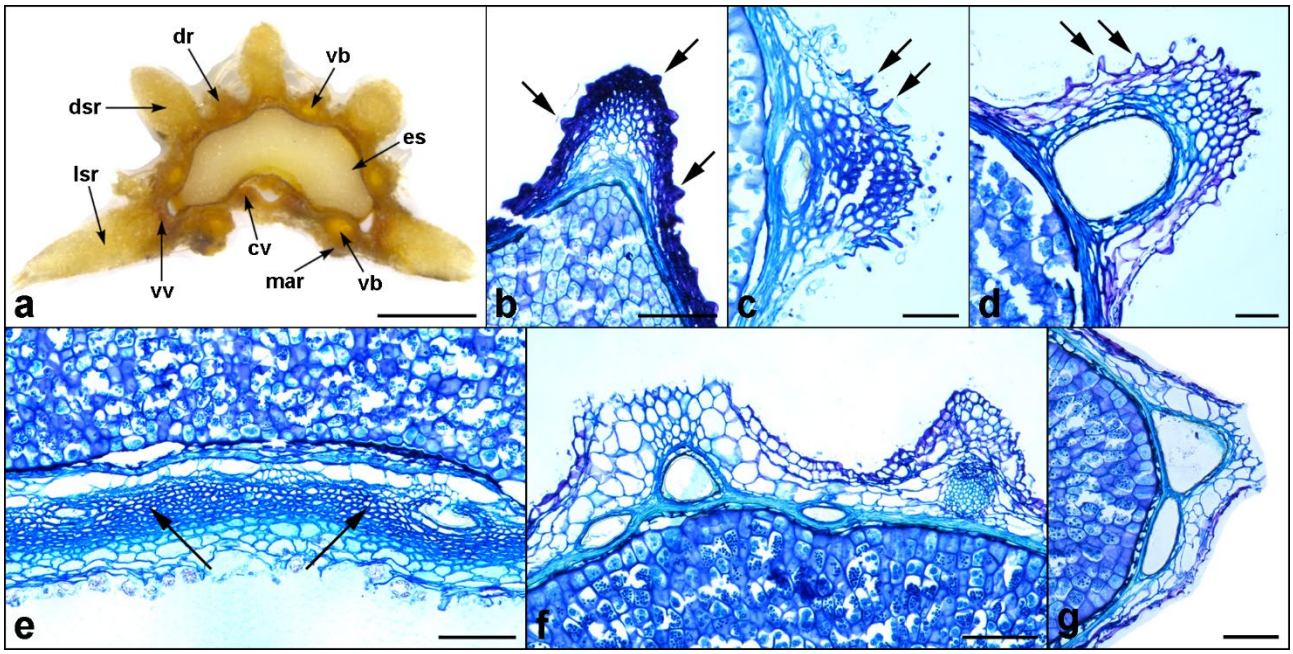

Figure 7. Selected distinct features or abnormalities found in the mericarps of *Daucus* and related taxa. (**a**) Abnormal mericarp of *D. littoralis* with additional dorsal primary and secondary ribs; (**b**) tubercles (arrows) on the exocarp of *D. aureus*; (**c**) characteristic exocarp cells with triangular appendages (arrows) covering the primary ribs of *D. glochidiatus* and (**d**) *D. sahariensis*; (**e**) close-up on the commissural side of *Orlaya daucoides* mericarps showing a hypendocarp (arrows); (**f**,**g**) additional smaller vallecular vittae in the cultivated carrot mericarps. Abbreviations: cv, commissural vitta; dr, dorsal primary rib; dsr, dorsal secondary rib; es, endosperm; lsr, lateral secondary rib; mar, marginal primary rib; vb, vascular bundle; vv, vallecular vitta. Scale bars: 1 mm (**a**); 50 μm (**c**,**d**); 100 μm (**b**,**e**–**g**).

The fruit wall (pericarp) of the investigated taxa had a typical structure of three layers: exocarp, mesocarp, and endocarp (see Figure 1), varying in thickness from 28 to 132 μm (Table 2). The single-layered exocarp consisted of small, thick-walled cells, usually flattened rectangular or more or less isodiametric in shape (Figure 1), but some exceptions were also found. In *D. aureus*, the exocarp was covered with numerous tubercles (Figures 3a and 7b), whereas, in *D. glochidiatus* and *D. sahariensis*, the part of the exocarp that covered the secondary ribs was composed of cells with triangular appendages (Figure 7c,d). The mesocarp consisted of a few to several layers of irregular thin-walled parenchymatic cells, typically larger than the exocarpic cells (Figure 1). The endocarp was a single compressed layer of somewhat lignified cells that usually adhered tightly to the seed coat (Figure 1). Regarding deviations in the pericarp structure among the outgroup taxa, we observed that the exocarp of *T. arvensis* was covered with numerous tubercles (Figures 3n and 6e), whereas the fruits of both *Orlaya* species were characterized by the presence of a hypendocarp, i.e., the inner fibrous mesocarp consisting of several layers of lignified fibers (Figure 7e).

Vallecular vittae were typically well developed in most members of *Daucus* and were triangular or ovate in shape (Figures 4–6); only *D. aureus* was devoid of these structures (Figure 6a). Among all taxa, *D. rouyi* exhibited the largest vallecular vittae (168 μm), whereas *D. muricatus* had the smallest (33 μm) (Table 2). The largest commissural vittae were found in *O. daucorlaya* (329 μm), and the smallest were found in *D. glochidiatus* (53 μm). Generally, each secondary rib enclosed one vitta; however, some variations were observed in carrots in which one or two additional smaller vittae—alongside the larger ones—were sometimes noticed (Figure 7f,g). All taxa, except for *D. aureus*, always had two commissural vittae that were ovate or compressed ovate in shape. Among the outgroups, the number and arrangement of both vallecular and commissural vittae were the same as in *Daucus*.

All *Daucus* taxa had a single compact vascular bundle embedded in the mesocarp below each primary rib. In *D. aureus*, however, the vasculature in the marginal primary ribs was connected in the commissure, forming a distinct M-shaped vascular bundle (Figure 6a). The size of the vascular bundles was more or less similar between the ribs of a given accession, except for *D. littoralis*, whose vascular bundles in the marginal primary ribs were distinctly larger than those in the dorsal primary ribs (Figure 6b). Among the outgroups, the most distinct differences in vasculature were flattened and elongated vascular bundles in the primary ribs of *C. platycarpos* (Figure 6d); the fruits of this taxon were also characterized by the presence of collenchyma in the secondary ribs.

In almost all taxa, the endosperm (commissural side) was flat or more or less concave, except for *C. platycarpos* (Figure 6d) and *T. arvensis* (Figure 6e), whose endosperm was mushroom-like grooved; *C. platycarpos* had strongly revolute margins.

4. Discussion

Traditionally, the taxonomic classification of the family Apiaceae has relied on the morpho-anatomical features of the fruits. However, many of the relationships inferred from this approach appear to be incongruent when confronted with molecular evidence. This is due to the high level of homoplasy among the fruit characteristics, which can be partially explained by selection [44]. Generally morphological characteristics are greatly affected by environmental factors [45–47]. Nevertheless, fruit characteristics can still provide useful information to support or supplement conclusions drawn from molecular data [27,32,44,48–50].

Here, we explored the morphology and anatomy of fruits in 13 *Daucus* and four closely related non-*Daucus* taxa. The results revealed a wide range of variation across the investigated taxa in terms of fruit size, shape, and weight, as well as fruit surface sculpturing and some anatomical characteristics. Thus, we pointed out several diagnostically valuable features of some of the *Daucus* taxa that we discuss below.

The morphometric characteristics and weights of the fruits differed significantly among the taxa (Table 1), which can be helpful—to some extent—in distinguishing between them. However, intra(sub)specific variations may occur in this regard, as observed here for cultivated carrot accessions. Moreover, in many cases, the quantitative values overlapped, which makes these data of limited taxonomic value. Therefore, the micromorphological features of the fruit surface, as well as fruit anatomy, appear to be more advantageous for distinguishing species.

Exocarp cell shape, exomesocarp protuberances, and cuticles are those components that contribute to fruit surface sculpturing, often providing taxonomically useful data [51]. In our study, as revealed by SEM, most of the investigated *Daucus* taxa had a rugose type of ornamentation, which can also be found, for instance, in *Ferula dshizakensis* [41] or some species of *Pimpinella* [52]. In *D. rouyi*, ribbed–striate fruit surface ornamentation was observed. This sculpturing pattern has also been reported, for example, in a few members of *Grammosciadium* [53] and *Pimpinella ibradiensis* [52]. Four *Daucus* taxa were characterized by the presence of tubercles, of which only *D. aureus* was covered on the entire surface of the mericarp, whereas *D. guttatus*, *D. littoralis*, and *D. muricatus* had only tuberculate secondary ribs. As for the exocarp cell shape (not visible by SEM), only *D. glochidiatus* and *D. sahariensis* were marked by the presence of distinct exocarp cells with triangular appendages that covered the surface of the secondary ribs; cells of this shape are characteristic of, for example, *Alepidea serrata* var. *serrata* [51]. Nonetheless, although the micromorphological characteristics of fruit surfaces have proven to be of taxonomic value, the application of these traits is difficult due to the lack of generally accepted terminology [54].

Species of *Daucus* and *Orlaya* (subtribe Daucinae), as well as *Caucalis* and *Torilis* (Torilidinae), are characterized by the presence of prominent secondary ribs, which is an almost unique trait among the members of these two subtribes and the genus *Artedia* [35,50]. In *Daucus*, the secondary ribs form spines or wings, the presence of which is a

distinct adaptation to seed dispersal by epizoochory (animal-mediated dispersal) or anemochory (wind-mediated dispersal). The genus *Daucus* has traditionally comprised only spiny-fruited species [5]; however, following a recent taxonomic revision by Banasiak et al. [11], numerous species with winged or obsolete fruits have been included in the genus. However, fruit appendages are characterized as highly homoplastic and are, thus, of limited utility in delimiting monophyletic groups [11,15].

The number and arrangement of both vallecular and commissural vittae within the pericarp are often of great taxonomic importance in Apiaceae. These secretory canals, located also in roots, stems, and leaves, are responsible for the specific odors of Apiaceae species as they contain essential oils, mucilage, gums, or resins [1], some of which are toxic to insects [55]. In our study, all taxa but one (*D. aureus*) had six vittae per mericarp: one below each secondary rib and two in the commissure, which is a common feature in most genera of Daucinae and Torilidinae [25]. Although we observed some variations in this regard in the cultivated carrot accessions that rarely had additional smaller vittae, these were presumably dwarf vittae, which could also be found, for instance, in *Apium graveolens* [56] or in many members of the Heteromorpheae tribe [57]. However, the size of the vittae seems to be more useful since this feature varied between many taxa.

In *Daucus* and related taxa, each mericarp had five vascular bundles—three in the dorsal primary ribs and two in the marginal primary ribs—as in almost all other members of Apiaceae. However, some exceptions to this pattern were found, for instance, in *Choritaenia capensis* [58] or *Cryptotaenia canadensis* [59], characterized by having seven vascular bundles, of which five were located on the dorsal side and two on the commissural side of the mericarp.

A lignified endocarp, composed of one layer of compressed and elongated cells, was present in all of the investigated *Daucus* taxa. De Miranda et al. [60] evidenced the process of lignin deposition in the endocarp cells of carrot fruit, along with their development, and reported that this process begins 21 days after anthesis.

Although the results showed considerable variations in the fruit morpho-anatomical characteristics, these variations were not sufficient enough to distinguish all of the investigated taxa. Exclusively on the basis of fruit characteristics, the most easily distinguishable taxon among *Daucus* was *D. aureus*, as it was characterized by several unique traits, i.e., entirely tuberculate fruit surface, lack of vittae, and distinct M-shaped vascular bundle on the commissural side. The partially tuberculate taxa (*D. guttatus*, *D. littoralis*, and *D. muricatus*) were distinguished by the length and weight of the mericarps, as well as by the features of their vascular bundles. The two taxa with characteristic exocarp cells with triangular appendages (*D. glochidiatus* and *D. sahariensis*) were differentiated according to the size of their vittae. In the case of *D. carota* subspecies, *D. carota* subsp. *capillifolius* differed from carrot accessions (subsp. *sativus*) by means of its mericarp length and oblong shape. *Daucus rouyi* was the only wing-fruited taxon in our sample. The remaining *Daucus* taxa (*D. syrticus*, *D. conchitae*, *D. involucratus*, *D. pusillus*, and the cultivated carrot) were morphologically and anatomically very similar to each other; thus, we were unable to unambiguously separate them.

5. Conclusions

This study provides detailed information on the morphology and anatomy of fruits from 13 *Daucus* and four closely related non-*Daucus* taxa. The results showed a wide range of variation in the fruit morpho-anatomical characteristics across the investigated taxa, as well as revealed several diagnostically valuable features of the fruits. For *Daucus*, the observed differences included the fruit size, shape (from ellipsoid to oblong), and weight, as well as the fruit surface sculpturing and some anatomical characteristics, i.e., the presence/absence and size of vittae, pericarp thickness, and the shape of exocarp cells. This study broadens the knowledge of the fruits of *Daucus* and may be useful for future taxonomical research on the genus and its close relatives.

However, to gain better insight into the relationships among the genus *Daucus*, further studies with a broader sample, including the remaining members of the genus, are needed.

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– przeprowadzeniu znacznej części doświadczeń;

– analizie statystycznej uzyskanych wyników;

– interpretacji, wizualizacji i dyskusji wyników;

– sformułowaniu wniosków;

– przygotowaniu pierwszej wersji manuskryptu;

– przygotowaniu i edycji ostatecznej wersji manuskryptu;

– opracowaniu odpowiedzi na uwagi recenzentów oraz wprowadzeniu stosownych poprawek do manuskryptu;

– pozyskaniu finansowania na badania i publikację artykułu;

– pełnieniu funkcji autora korespondencyjnego.

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Bydgoszcz, 3.01.2023 r.

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Wydział Rolnictwa i Biotechnologii
Politechnika Bydgoska im. Jana i Jędrzeja Śniadeckich

Oświadczenie o współautorstwie

Oświadczam, że w pracy

> Kadluczka D., Sliwinska E., Grzebelus E. 2022. Combining genome size and pollen morphology data to study species relationships in the genus *Daucus* (Apiaceae). *BMC Plant Biology*, 22, 382. DOI: https://doi.org/10.1186/s12870–022–03743–1

mój indywidualny udział w jej powstaniu polegał na:

– opracowaniu metodologii analiz cytometrycznych;

– przeprowadzeniu analiz cytometrycznych i interpretacji jej wyników;

– uczestniczeniu w opracowaniu końcowej wersji manuskryptu.

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prof. dr hab. inż. Elwira Śliwińska

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Wydział Biotechnologii i Ogrodnictwa
Uniwersytet Rolniczy w Krakowie

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mój indywidualny udział w jej powstaniu polegał na:

– opracowaniu koncepcji badań;

– opracowaniu metodologii do części badań;

– opiece i nadzorze merytorycznym nad realizowanymi zadaniami badawczymi;

– opracowaniu ostatecznej wersji manuskryptu

– pozyskaniu części finansowania na badania i publikację artykułu;

– pełnieniu funkcji autora korespondencyjnego.

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Uniwersytet Rolniczy w Krakowie

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– opracowaniu odpowiedzi na uwagi recenzentów oraz wprowadzeniu stosownych poprawek do manuskryptu;

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D. Kadłuczka

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Kraków, 03.01.2023

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