Waterfowl endozoochory: an overlooked long-distance dispersal mode for *Cuscuta* (dodder, Convolvulaceae)[1]

Mihai Costea[2, 7], Saša Stefanović[3], Miguel A. García[3], Susan De La Cruz[4], Michael L. Casazza[5], and Andy J. Green[6]

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[2]Department of Biology, Wilfrid Laurier University, Waterloo, Ontario N2L 3C5, Canada

[3]Department of Biology, University of Toronto Mississauga, Mississauga, Ontario L5L 1C6, Canada

[4]U.S. Geological Survey, Western Ecological Research Centre, San Francisco Bay Estuary Field Station, 505 Azuar Drive, Vallejo, California 94592, U.S.A

[5]U.S. Geological Survey, Western Ecological Research Centre, Dixon Field Station, 800 Business Park Drive, Dixon, CA 95620, U.S.A]

[6]Wetland Ecology Department, Estación Biológica de Doñana (EBD-CSIC), Sevilla 41092, Spain

[7]Author for correspondence (e-mail: mcostea@wlu.ca)

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ABSTRACT

Premise of study. Dispersal of parasitic *Cuscuta* (dodders) species worldwide has been assumed to be largely anthropomorphic because their seeds do not match any dispersal syndrome and no natural dispersal vectors have been reliably documented. However, the genus has a subcosmopolitan distribution and recent phylogeographic results have indicated that at least18 historical cases of long-distance dispersal (LDD) have occurred during its evolution. The objective of this study is to report the first LDD biological vector for *Cuscuta* seeds.

Methods. Twelve northern pintails (*Anas acuta*) were collected from Suisun Marsh, California and the contents of their lowest part of the large intestine (rectum) were extracted and analysed. Seed identification was done both morphologically and using a molecular approach. Extracted seeds were tested for germination and compared to seeds not subjected to gut passage to determine the extent of structural changes caused to the seed coat by passing through the digestive tract.

Key results. Four hundred and twenty dodder seeds were found in the rectum of four northern pintails: 411 seeds were identified as *C. campestris* and nine as most likely *C. pacifica*. The germination rate of *C. campestris* seeds after gut passage was 55%. Structural changes caused by the gut passage in both species were similar to those caused by an acid scarification.

Conclusions. Endozoochory by waterbirds may explain the historical LDD cases in the evolution of *Cuscuta* and suggest that current border quarantine measures may be insufficient to stopping spreading of dodder pests along migratory flyways.

Key words: *Anas acuta*; long-distance dispersal; ITS; morphology; northern pintail; parasitic plants; seeds; trnL-F.

"The dispersal of the Dodders to remote islands is very puzzling."

Ridley, 1930

Seeds of the parasitic plant genus *Cuscuta* (dodder) have been considered “unspecialised” or "non-adapted" because they lack a morphological dispersal syndrome (Ridley, 1930; Kuijt, 1969; Dawson et al., 1994; Costea and Tardif, 2006). Natural dispersal by wind (Lyshede, 1984) and water have been anecdotally suggested for a select number of species (reviewed by Dawson et al., 1994; Costea and Tardif, 2006), but there is no evidence to suggest that these vectors allow long-distance dispersal (LDD). No other natural dispersal vectors have been recognized for *Cuscuta* diaspores, although seeds have been repeatedly recorded in the diet of waterfowl (see discussion). Yet the genus has a subcosmopolitan distribution and its nearly 200 species inhabit a great variety of habitats ranging from cold-temperate to tropical, riparian to desert, coastal to high mountains, grasslands, sand-dunes, forests, saline, vernal pools, ruderal, and agricultural (Yuncker, 1932; Costea et al., 2015a). Recent phylogeographic results have indicated that a minimum of 18 remarkable LDD events have occurred in the diversification of *Cuscuta* at different taxonomic levels: subgeneric, specific, and varietal (Stefanović et al., 2007; García et al., 2014). These LDD events inferred phylogenetically likely occurred before the evolution of *Homo sapiens*, and until now all of them have been biologically inexplicable in view of the limited natural dispersal ability recognized for *Cuscuta* seeds. For example, the evolution of subg. *Grammica*, the largest infrageneric group of *Cuscuta* (ca. 150 sp.) distributed mostly in the Americas, most likely involved a transoceanic dispersal from South Africa to South America (Stefanović et al., 2007; García et al., 2014). LDD occurred subsequently from the North or South American *Grammica* clades to some islands and virtually to all the other continents; e.g., *C. gymnocarpa* and *C. acuta* to the Galapagos Islands (Costea et al., 2015b); *C. sandwichiana* to Hawaii; *C. tasmanica* and *C. victoriana* to Australia (Costea et al., 2013); *C. kilimanjari* to eastern Africa; *C. hyalina* to Africa and Asia (Costea and Stefanović, 2010); *C. chinensis* (var. *chinensis*; Costea et al., 2011) and *C. australis* to Asia (see more cases in García et al., 2014).

Numerous *Cuscuta* species are major global pests, capable of drastically reducing the yield of numerous agricultural/horticultural crops or invading natural ecosystems (Dawson et al., 1994; Parker and Riches, 1993). The long-distance movement of such dodder species has long been thought to take place exclusively via contaminated seeds of various crops or Asian herbal products (Beal, 1910; Knepper et al., 1990; Dawson et al., 1994; Costea and Tardif, 2006). As a result, most countries have adopted legislation measures for surveillance and quarantine at the border to prevent the introduction of foreign *Cuscuta* sp. within their territory (Costea and Tardif, 2006). It is therefore important to know if *Cuscuta* seeds can also undergo LDD via non-human vectors.

The objective of this short-note is to report for the first time the endozoochory of *Cuscuta* (dodder, Convolvulaceae) seeds by a migratory waterfowl (northern pintails *Anas acuta* L.; Anatidae), and to discuss the implications of this finding.

MATERIALS AND METHODS

As part of a broader study of seed dispersal by waterfowl in the San Francisco Bay area, 11 northern pintails (hereafter referred to as “pintails”), were collected from Wings Landing, Suisun Marsh, Solano Co., California (38°13'31.63"N, 122° 2'7.61"W). Pintails migrate from breeding grounds in Canada, Alaska and Russia to winter in this area (Miller et al., 2005). Four birds were collected on 25 Jan. 2015 and seven on 2 Feb. 2015. After collection, birds were kept in a cooler on wet ice and dissected within 12 h. Collection of pintails was carried out under the guidance of the U.S. Geological Survey, Western Ecological Research Center’s Animal Care and Use Committee with permits from California Department of Fish and Game (SCP #003855) and the U. S. Fish and Wildlife Service (MB #102896). The use of trade, product, or firm names in this publication is for descriptive purposes only and does not imply endorsement by the U.S. Government.

None of the pintail showed any signs of disease upon dissection. The lower alimentary canal from the small intestine to the cloaca was removed after sealing the external part of the cloaca with duct tape, and then placed in the fridge. Within one to four days of collection, the internal contents of the lowest part of the large intestine ("rectum" from hereon, 6–8 cm in length) were extracted by cutting off the intestine immediately below the caeca, then squeezing the contents into dechlorinated tap water. Being at the end of the digestive system, the rectum was selected for study to ensure that present seeds had completely survived the digestive process (Brochet et al. 2010). The sample was washed through an 85 µm sieve then placed in a petri dish for inspection under the binocular microscope. Seeds were removed, classified and counted. In order to test their viability, 80 similar morphologically seeds extracted from an individual labelled NOPI 29 were placed for germination on 3 Feb. 2015 and checked twice a week until 2 Apr. 2015 when the germination trial was terminated. Seeds were placed at room temperature (minimum 21°C and maximum 25°C) in a sunlit window on filter paper placed on top of a layer of cardboard soaked in distilled water inside petri dishes.

Another 20 seeds, similar morphologically to the ones used above, were kept dry and germinated on 15 May at the University of Toronto, Mississauga. The seven seedlings produced (six from NOPI 29 and one from a different bird labelled NOPI 30) were used to identify the dodder species. Five of these seedlings were identified through DNA barcoding, using ITS (nuclear) and *trnL-F* (plastid) DNA sequences. Seedlings were frozen in liquid nitrogen and pulverized using solid glass beads (3 and 6 mm; Fisher Scientific) and a mixer mill (MM 300, Retcsh GmbH; 1 min at 30 Hz). DNA extractions, polymerase chain reaction (PCR) reagents and conditions, and amplicon purifications followed the protocols detailed in Stefanović et al. (2007). Cleaned PCR products were sequenced at the McGill University and Génome Québec Innovation Centre (Canada). A total of four ITS and seven *trnL-F* sequences were analyzed and deposited in GenBank (accession numbers ######–######). Sequences were aligned manually using Se-Al v.2.0a11 (Rambaut, 2002) and compared with our database containing a large number of *Cuscuta* species from the subgenus *Grammica* used in our previous broad-scale phylogenetic analyses of this group (Stefanović et al., 2007; Stefanović and Costea, 2008) as well as more recent analyses targeting specifically the *Cuscuta pentagona/campestris* species group (Costea et al., 2015b). To further characterize newly obtained DNA sequences, we compared them with those deposited in Genbank using BLAST. The remaining sixth seedling of NOPI 29 was grown using *Plectranthus scutellarioides* (L.) R.Br. (Lamiaceae) as a host in the University of Toronto Mississauga greenhouse. At maturity, flowers of this dodder specimen were collected, dissected and examined to identify the species morphologically. Herbarium and spirit vouchers of this plant were deposited in the TRTE and WLU herbaria.

Morphology of all the seeds was initially surveyed with a Nikon SMZ1500 stereomicroscope. 30 of the 320 remaining *Cuscuta* seeds were rehydrated and examined to determine the extent of morphological and structural changes caused by their passing through the digestive tract of pintails. Subsequently, seeds were cut in half through the hilar region, perpendicular to the hilum scar, subjected to a hexamethyldisilazane (HMDS) treatment as an alternative method to critical point drying (Wright et al., 2011), mounted on specimen stubs, and coated with 30 nm of gold using an Emitech K550 sputter coater. Examination of the surface and seed coat structure was conducted with a Hitachi SU-1500 Scanning Electron Microscope (SEM) at 3 KV. Seeds were compared to those in a morphological database of *Cuscuta* seeds (Costea, unpublished) after a search of the dodder species present at Suisun Marsh (Vasey et al., 2012; Consortium of California Herbaria, 2015).

After the identification of the seeds retrieved from the pintails (see Results), typical dodder seeds of the same species that had not been subjected to gut passage were prepared and examined as indicated above from the following herbarium specimen deposited in the herbarium of Wilfrid Laurier University (WLU): *Cuscuta campestris* Yunck. U.S.A., California, Sonoma Co., Sep. 2007, *Cadman* et al. *2832*; Riverside Co., 28 Jul. 1994, *Sanders 15174* (UCR); San Bernardino Co., 1 Sep. 2000, *Provance 2227B* (UCR). *Cuscuta pacifica* Costea and M.A Wright. California: Humboldt Co., 28 Aug. 1941, *C.C. and S.K. Harris 1175* (DAO); Solano Co., 8 Dec. 1959, *Crampton 5472* (CAS). Oregon: Lincoln Co., 30 Jul. 1995, *Halse 4961* (NY).

RESULTS

Of the 11 pintails collected from Suisun Marsh, four of them had intact *Cuscuta* seeds in the rectum. Two individuals had a single seed, one individual (NOPI 30) had six seeds, and the fourth individual (NOPI 29) had 412 seeds. The majority of *Cuscuta* seeds were small, 0.7–1.2 mm long. However, nine of the *Cuscuta* seeds examined from NOPI 29 were larger, 1.4–1.9 mm long, indicating that two different dodder species were ingested by this individual.

Barcoding showed that the small dodder seeds found inside the rectum of pintail belong to *C. campestris* (field dodder). All sequences generated in this study (four ITS and seven *trnL-F*) were either identical to or had ≤ 2bp differences compared with those of *C. campestris* obtained for our previous studies (Stefanović et al., 2007; Stefanović and Costea, 2008; Costea et al., 2015b). Also, the BLAST search of online DNA databases showed that the sequences from the seedlings are compatible with *C. campestris*, with the highest scores having 100% query coverage and 99–100% identity with *C. campestris* and other closely related members of the *C. campestris/pentagona* species group. The same species identity, *C. campestris*, was obtained through the morphological examination of dissected flowers produced by the mature *Cuscuta* plant grown in the greenhouse. The nine larger seeds found in NOPI 29 were identified morphologically as most likely belonging to *C. pacifica* (Pacific salt-marsh dodder).

The 80 seeds tested for viability belonged to *C. campestris*: 23 seeds germinated by 9 Feb. (28.75%), and 44 by 23 Mar. (55%). Germination of the nine seeds of *C. pacifica* was not tested because of their insufficient number. The passage through the digestive tract of pintails modified significantly the structure of the seed coat in both *Cuscuta* species (Fig. 1). In *C. campestris*, the seed coat maintained its integrity, remaining attached to the endosperm (Fig. 1 a–c); in *C. pacifica* the seed coat fragmented and detached from the endosperm (Fig. 1 d–e). In both species, the epidermis with dome-like cells, which is always present in seeds not subjected to gut passage (Figs. 1 j, p), was entirely stripped out. The external palisade layer, also characteristic to *Cuscuta* seeds (Figs. 1 k, r), was entirely digested in *C. pacifica* (Figs. 1 d–e; m–n), and partially or totally eliminated in *C. campestris* (Figs. 1 a–c; g–h). Remnants of the external palisade layer may persist on irregular surfaces in *C. campestris* (Fig. 1a), but most often it can be found only in concave areas or in the hilar zone (Figs. 1b, f). Thus, in both dodder species, after the digestion process, the testa was reduced to the internal palisade layer, which was brought to the surface of seeds (Figs. 1 g, m; h, n). Also, in both species, the hilum, which is responsible for imbibition in *Cuscuta*, becomes entirely exposed: a nearly invisible line in *C. campestris* (Fig. 1 f; compare with 1 i), and more conspicuous in *C. pacifica* (Fig. 1 l; compare with 1 o).

DISCUSSION

Zoochory has not previously been proposed as a dispersal mode for *Cuscuta*, but in retrospect the existing literature provides much support for our proposal since *Cuscuta* sp. seeds have already been reported in the diets of several species of migratory waterfowl (Cottam, 1939; Martin and Uhler, 1939; Chamberlain, 1959; Goodrick, 1979) and shorebirds (Beltzer, 1991). However, none of these studies identified the *Cuscuta* species involved or tested the viability of passed seeds. In this study, the seeds of *C. campestris* retrieved after gut passage were viable and germinated at rates comparable to those reported for scarified seeds of this species at 21–24°C (e.g., Hutchison and Ashton, 1980; Benvenuti et al., 2005). Dormancy of *Cuscuta* seeds is physical, imposed by the impermeable seed coat with two palisade layers (e.g. Hutchison and Ashton, 1979; Lyshede, 1984; Jayasuriya et al., 2008). The structural changes reported after gut passage, especially the fragmentation of the seed coat in *C. pacifica*, are similar to those observed in *Cuscuta* after sulfuric acid scarification (Costea, unpublished). Although we only found small numbers of seeds in three of the four ducks where *Cuscuta* was present, we only inspected a short section of the hindgut which holds a very small proportion of seeds carried through the entire digestive system (Brochet et al. 2010).

Waterbirds provide a major ecosystem service by dispersing plants that lack a fleshy fruit and hence are not dispersed by frugivores (Green and Elmberg, 2014). The potential of migratory waterbirds to disperse plants over long distances and to oceanic islands has long been recognized (Darwin, 1859; Proctor, 1968; Carlquist, 1967). Experimental and field studies suggest waterfowl are major but largely overlooked vectors for a broad range of wetland and terrestrial plants, including many species with "non-adapted" seeds like *Cuscuta* (Brochet et al., 2009, 2010; Soons et al., 2016). Dodder seeds or fruits may be washed into wetlands by rainfall, making them available to dabbling ducks such as pintail. Modelling confirmed that dabbling ducks readily disperse seeds over hundreds of kilometres or more (Viana et al., 2013). Among other Convolvulaceae with subcosmopolitan distribution, Proctor (1968) showed experimentally that viable seeds of *Convolvulus arvensis* can be retained in the gut of shorebirds for up to 144 h, which would be enough to cross the Pacific Ocean (Gill et al., 2009). Thus, as previously suggested for fleshy-fruited plants (e.g., Popp et al., 2011), our findings indicate that waterbirds or shorebirds may explain the historical LDD events that took place in the evolution of *Cuscuta*.

Pintails wintering in California, Mexico, Caribbean, and Central America undertake rapid long-distance migratory movements northwards to various parts of North America and Russia (Miller et al., 2005; Arzel et al., 2006). Similarly, pintails wintering in the Mediterranean Basin and Africa or in Southeastern Asia (e.g., Japan) migrate to various northern areas of Europe and Asia (Arzel et al., 2006; Hupp et al., 2006). Although northern pintail migration routes are usually not transoceanic, vagrants occasionally cross the Atlantic or Pacific (e.g., Flint et al., 2009). However, this particular duck species is probably not the dispersal vector involved in most historical LDD events that took place in the evolution of *Cuscuta*. One possible exception is *C. sandwichiana,* which is part of a North American clade but is endemic to Hawaii (García et al., 2014; Costea et al., 2015a), where pintails winter regularly (e.g., Udvardy and Engilis, 2001). Endozoochory by shorebirds is the most likely explanation for other historical LDD events (Carlquist, 1967).

Cuscuta pacifica is the typical dodder of saline tidal marshes on the Pacific Coast (Costea et al., 2009), including at Suisun Marsh (Barbour et al., 2007; Vasey et al., 2012, referred to as "*C. salina*"). *Cuscuta subinclusa*, a closely related species (Costea et al., 2009), which is also present in the area (Vasey et al., 2012; Consortium of California Herbaria, 2015), has similar seeds morphologically (Costea et al., 2006) but it grows mostly on shrubs and trees. The seeds of all three *Cuscuta* species are enclosed in indehiscent fruits, which are usually persistent on the hosts in dense infructescences until the spring. For these reasons, it is more likely that the large seeds belong to *C. pacifica* which, like *C. campestris*, parasitize herbaceous hosts (Costea et al., 2009) and their fruits are more accessible to pintails feeding at ground level. Although this is not one of the LDD cases highlighted by García et al. (2014), the dispersal of *C. pacifica* over 2000 km of coast from British Columbia, Canada, via Washington, Oregon, and California in the U.S.A., to Baja California in Mexico, may have involved pintails or other migratory waterbirds.

Cuscuta campestris is perhaps the most common weedy dodder worldwide (Costea et al., 2015b), and its ubiquitous presence has until now been considered to be explained solely as a result of human dispersal through contaminated seed crops. Our findings suggest that avian endozoochory may have also contributed to the widespread distribution of this species. For example, in this study the presence at Suisun Marsh shows that *C. campestris* movement is not necessarily linked to agricultural practices. Ducks have probably dispersed field dodder (and perhaps other species) within North America, Europe and Asia. It has been recently reported (Costea et al., 2015b) that *C. gymnocarpa* Engelm., which is endemic to the Galapagos, is in fact a form of *C. campestris* that has evolved in the archipelago after a LDD event from the mainland. Thus, the possibility of endozoochory opens a new direction of research in the ecology and biogeography of *Cuscuta*. Finally, the potential for endozoochory reported here suggests that enforcement of the current border quarantine measures will not be sufficient to completely curtail the international movement of field dodder and other *Cuscuta* pest species.

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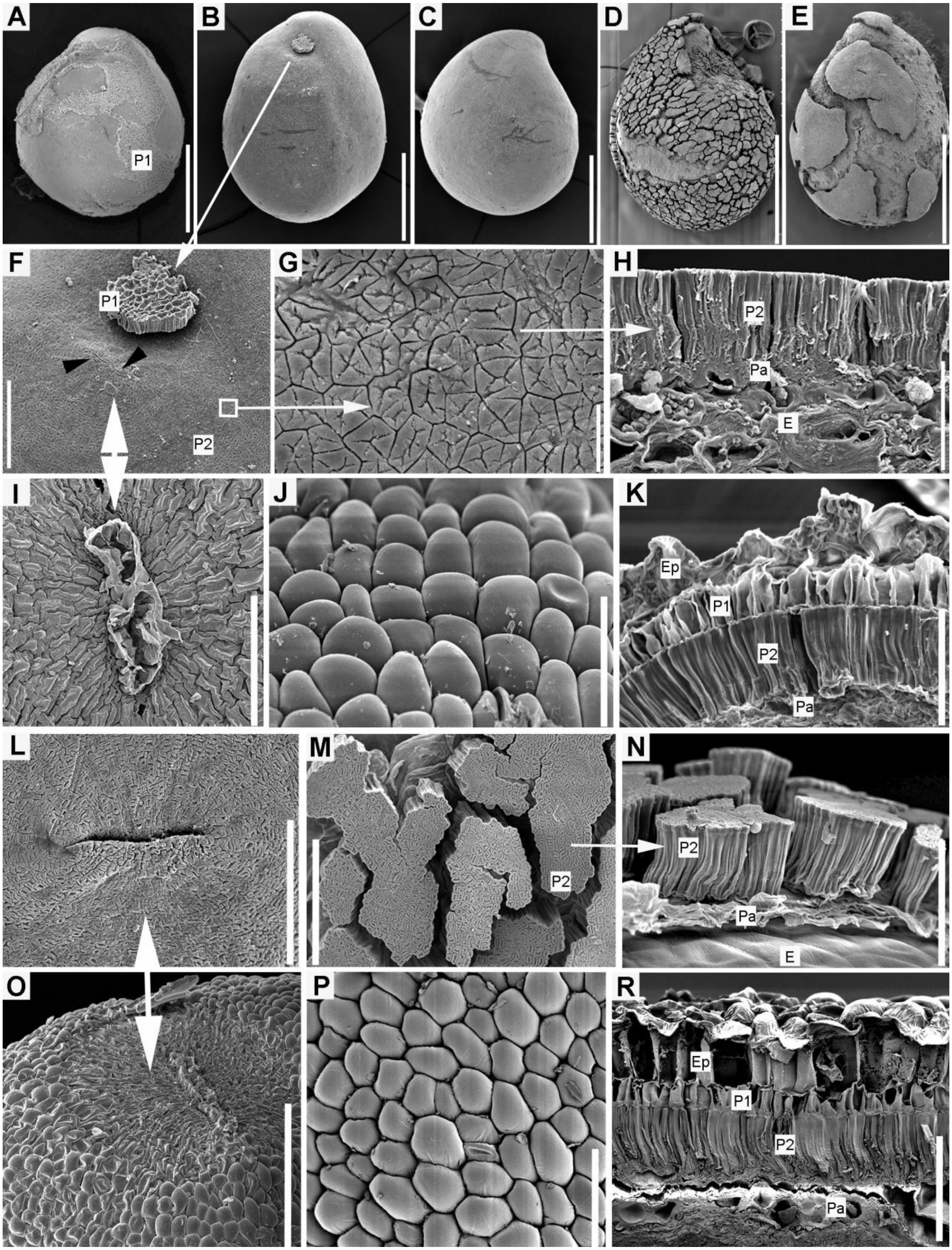
FIGURE LEGENDS

Fig. 1. Morphological and structural changes caused by the passing of *Cuscuta* seeds through the digestive tract of northern pintail. (a–c). Passed seeds of *C. campestris*; note that the external palisade layer persisted on irregular portions of the seed (a), in the hilum area (b), or it was completely eliminated. (d–e). Passed seeds of *C. pacifica*: the seed coat is fragmented and the external palisade layer was entirely removed. (f–h). Surface details and anatomy of seed coat in passed seeds of *C. campestris*. (f). Hilum area (black arrows delineate the hilum). (g–h). Sclereids of the internal palisade layer were brought to the surface of the seed coat because the epidermis and external palisade layer were entirely digested. (i–k). Surface details and anatomy of seed coat in *C. campestris* seeds not subjected to gut passage. (i). Hilum is quite visible. (j). Epidermis with dome-like cells. (k). Anatomy of complete seed coat (with epidermis and external palisade layer). (l–n). Surface details and anatomy of seed coat in passed *C. pacifica* seeds: hilum (l) is more visible than in *C. campestris*; internal palisade layer fragments and seed coat detaches from endosperm (m–n). (o–r). Surface details and anatomy of seed coat of *C. pacifica* seeds not subjected to gut passage. P1 = external palisade layer; P2 = internal palisade layer; Pa = parenchyma; Ep = epidermis; E = endosperm. Scale bars a–c, 0.5 mm; d–e, 1 mm; f, i, l, m, o, 100 μm; h, j, k, n, p, r, 50 μm; g, 10 μm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