

RESEARCH ARTICLE

New intergeneric orchid hybrid found in Romania × *Pseudorhiza nieschalkii* (Senghas) P.F.Hunt nothosubsp. *sicolorum* H.Kertész & N.Angheliescu, 2020

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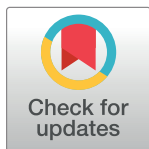
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Abstract

We describe the first reported intergeneric, which naturally occurs between two subspecies belonging to different genera, *Dactylorhiza fuchsii* subsp. *sooana* (genus *Dactylorhiza*) and *Pseudorchis albida* subsp. *tricuspis* (genus *Pseudorchis*), as × *Pseudorhiza nieschalkii* (Senghas) P.F.Hunt nothosubsp. *sicolorum* H.Kertész & N.Angheliescu, 2020. The hybrid was found and digitally photographed for the first time by Hajnalka Kertész in June, 2020, within Terra Siculorum, in one of the Natura 2000 protected areas, known as Harghita Mădăraș, ROSCI00090. Following detailed morphometric analysis using 67 characters and molecular karyological analyses, we identified this unique specimen as an intergeneric hybrid, new to science. The hybrid, an F₁ generation plant, most likely representing a single intergeneric pollination event, is phenotypically intermediate between its parental species in most of the characters scored, but it significantly closely resembles *Pseudorchis albida* subsp. *tricuspis* parent. Since several individuals of the parental species occurred in near proximity, within 1–10 meters distance, we suggest that the production of this hybrid required a minimum travel distance of ca 1–10 meters, by the pollinators and frequent exchange of pollen between the parental species was very likely. The parental species and the hybrid, which display a considerable synchronicity in their flowering time, overlap in the pollinator community, sharing various species of Hymenoptera and Diptera, very abundant in the heathland. This Terra Siculorum hybrid is thus best described as a rarely occurring intergeneric hybrid that shows strong *Pseudorchis albida* subsp. *tricuspis* parental dominance in inheritance patterns.

Introduction

Over millions of years of evolution, hybridization had a major role in shaping the history of life on earth. The evolutionary history of a population is reflected in the genetic variation of its genomes. In natural populations, hybridization can act as an evolutionary engine by overcoming the reproductive barriers between populations.

Hybridisation is the crossing of two genotypically different parents, parent generations $P_1 \times P_2$. The genes from P_1 & P_2 combine in the first resulted generation, named F_1 [1]. They will be present in the hybrid genotype and can be dominant, recessive or intermediate [2]. A new hybrid lineage is formed through parental genome mixing. The totality of all successful hybrid types that originate of the crossing of two parental taxa (natural species, not of hybrids) is called a nothotaxon. Nothotaxons may further progress as nothogenus, nothospecies or notho-subspecies. Hybridization is widespread, but the generation of a unique, natural hybrid lineage to occur is likely very rare. New hybrid lineages must establish reproductive isolation and a unique ecological niche in order to overcome genetic mixing and competition from parental species [3]. As a result, hybridisation was shown to have a significant role in speciation, generating new species with better genetic, adaptive variation [4].

We document the first reported natural occurrence of an intergeneric hybrid between the rare subspecies, *Dactylorhiza fuchsii* subsp. *sooana* and the alpine species *Pseudorchis albida* subsp. *tricuspis*, the only species present within the hybrid's close proximity. This previously undescribed allopolyploid, named as × *Pseudorhiza nieschalkii* (Senghas) P.F.Hunt nothosubsp. *sicolorum* H.Kertész & N.Anghelescu, was discovered by biologist Hajnalka Kertész, on the 30th of June, 2020, during a field trip. The genetic constitution as revealed by the subsequent hybrid karyotyping confirms that its parents are the diploid *Dactylorhiza fuchsii* subsp. *sooana* (genus *Dactylorhiza*) and *Pseudorchis albida* subsp. *tricuspis* (genus *Pseudorchis*), the diploid (allopolyploid) genome of the hybrid counting $2n = 40$ chromosomes.

The hybrid was found in one of the most fascinating heathlands in Tierra Sicolorum, one of Natura 2000 protected areas known as Harghita-Mădăraş, very famous for its rich orchid flora. In recent years, our studies encountered several orchid species, usually in substantial numbers such as *Dactylorhiza maculata* (L.) Soó (Heath Spotted-orchid), *Dactylorhiza incarnata* (L.) Soó (Early Marsh-orchid), *Platanthera bifolia* (L.) Rich. (Lesser Butterfly-orchid), *Dactylorhiza cordigera* subsp. *sicolorum* (Soó) Soó (Heart-flowered Marsh-orchid), *Gymnadenia frivaldii* Hampe ex Griseb. (Frivald's Gymnadenia), *Pseudorchis albida* (L.) Á.Löve & D.Löve (Small White Orchid), *Pseudorchis albida* subsp. *tricuspis* (Beck) E. Klein (Trilobate White Pseudorchis), the dwarf *Dactylorhiza fuchsii* subsp. *psychrophila* (Schltr.) Holub (Cold-loving Dactylorhiza) and rarest and most elusive *Dactylorhiza fuchsii* subsp. *sooana* (Borsos) Borsos (Soó's Spotted-orchid), all included in our studies. Due to the unusual weather conditions and specific microclimate of the area, which is mainly characterised by cool and rainy summers, most of these orchid species occur in substantial numbers at Harghita-Mădăraş heathland.

The opportunities for this particular hybridisation event to occur are extremely limited, as the parental species *Dactylorhiza fuchsii* subsp. *sooana* and *Pseudorchis albida* subsp. *tricuspis* are rather rare and have contrasting habitat preferences and geographic distributions. To date, Harghita-Mădăraş heathland is the only location where the two subspecies are known to occur in significant, sympatric populations.

This hybrid nothosubspecies belongs to the nothospecies × *Pseudorhiza nieschalkii* (Senghas) P.F.Hunt (hybrid formula: *Dactylorhiza fuchsii* × *Pseudorchis albida*), which is a member of the subtribe Orchidinae Dressler & Dodson, 1960 / Verm., 1955, tribe Orchideae Dressler & Dodson, 1960 / Verm. 1977, subfamily Orchidoideae Lindl., 1826, family Orchidaceae Juss., 1789. The orchid hybrid, × *Pseudorhiza nieschalkii*, was first described in 1968, by German

botanist and orchidologist Karlheinz Senghas (1928–2004) and named × *Dactyleuorchis nieschalkii* Senghas [5]. In 1971, British botanist Peter Francis Hunt (born 1936) gave its current name, × *Pseudorhiza nieschalkii* (Senghas) P.F.Hunt [6].

× *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* is the first intergeneric hybrid between *Dactylorhiza* and *Pseudorchis* genera, ever mentioned in Romania and the first intergeneric hybrid between *Dactylorhiza fuchsii* subsp. *sooana* and *Pseudorchis albida* subsp. *tricuspis*, ever mentioned in literature. Therefore, we strongly propose this hybrid as new to science (nothosubsp. nov.) and consequently, as a new addition to the Romanian flora.

Materials and methods

The root-tip collection from a natural setting was allowed by the permission given by Agenia Naională pentru Arie Naturale Protejate Serviciul Teritorial Harghita (National Agency for Natural Protected Areas Harghita Teritorial Service) Nr. 724/ST.HR./12.10.2020.

Location description

The natural area Harghita—Mădăraş is located in the central-eastern part of Harghita County, on the administrative territory of Vlăhiţa and those of the communes Căpâlniţa, Cârţa, Dăneşti, Mădăraş, Racu, Siculeni, Suseni and Zetea [7]. The area was declared a site of Community importance by Order of the Ministry of Environment and Sustainable Development No. 1964 of 13 December 2007 (on the establishment of the protected natural area regime of Sites of Community Importance, as an integral part of the European ecological network Natura 2000 in Romania) and covers an area of 13,373 hectares. Harghita—Mădăraş site is thus designated to protect its biodiversity and preserve the wild flora and fauna within its territory, as well as other natural habitats of community interest located in the protected area. It is located at an altitude between 1500 and 1800 m [8–10], (Fig 1).

The site is a natural area covered by deciduous, coniferous and mixed forests, natural meadows, heathlands, bogs (peat bogs) and steppes framed within the alpine bioregion of the Harghita Mountains range. It contains a rich hydrographic network which consists of several lakes and watercourses [11, 12].

The vegetation of this wetland reserve was dominated by characteristic herbaceous swampy species. *Sphagnum* L. moss was generally abundant, along with various ericaceous shrubs such as *Abies alba* Mill. (Fir), *Picea abies* (L.) H.Karst. (Spruce), *Taxus baccata* L. (Yew), *Fagus sylvatica* L. (Beech), *Quercus petraea* (Matt.) Liebl. (Oak), *Sorbus aucuparia* L. (Mountain Bark), *Fraxinus excelsior* L. (Ash), *Pinus mugo* Turra (Juniper), *Juniperus communis* L. (Juniper), *Sambucus nigra* L. (Sack), *Corylus avellana* L. (Hazel), *Rubus idaeus* L. (Raspberry), *Rosa canina* L. (Rosehip), *Rubus vestitus* Weihe (Blackberry), *Vaccinium myrtillus* L. (Blueberry), *Epilobium nutans* F.W.Schmidt (Willow Herb), *Veronica fruticans* Jacq. (Veronica), *Campanula patula* subsp. *abietina* (Griseb. & Schenk) Simonk. (Bells), to name only a few [13–15].

This unique hybrid, × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum*, was located in a full sun, grassy area, adjacent to a forest margin that covered approximately 1.2 square kilometres. It consisted mainly of a wetland reserve, formed on nutrient poor, acidic to neutral peat substrate. The surface of the soil was moist but not water logged (marshy meadow), mainly covered in *Sphagnum* L. moss.

D. fuchsii subsp. *sooana* occurred in moderate numbers and it was found to grow immediately adjacent to the hybrid. Its numbers probably encountered 15–20 plants. The hybrid and *D. fuchsii* subsp. *sooana* parental population were growing within the marshy area of the swamp. The distances between the hybrid and *D. fuchsii* subsp. *sooana* plants were relatively

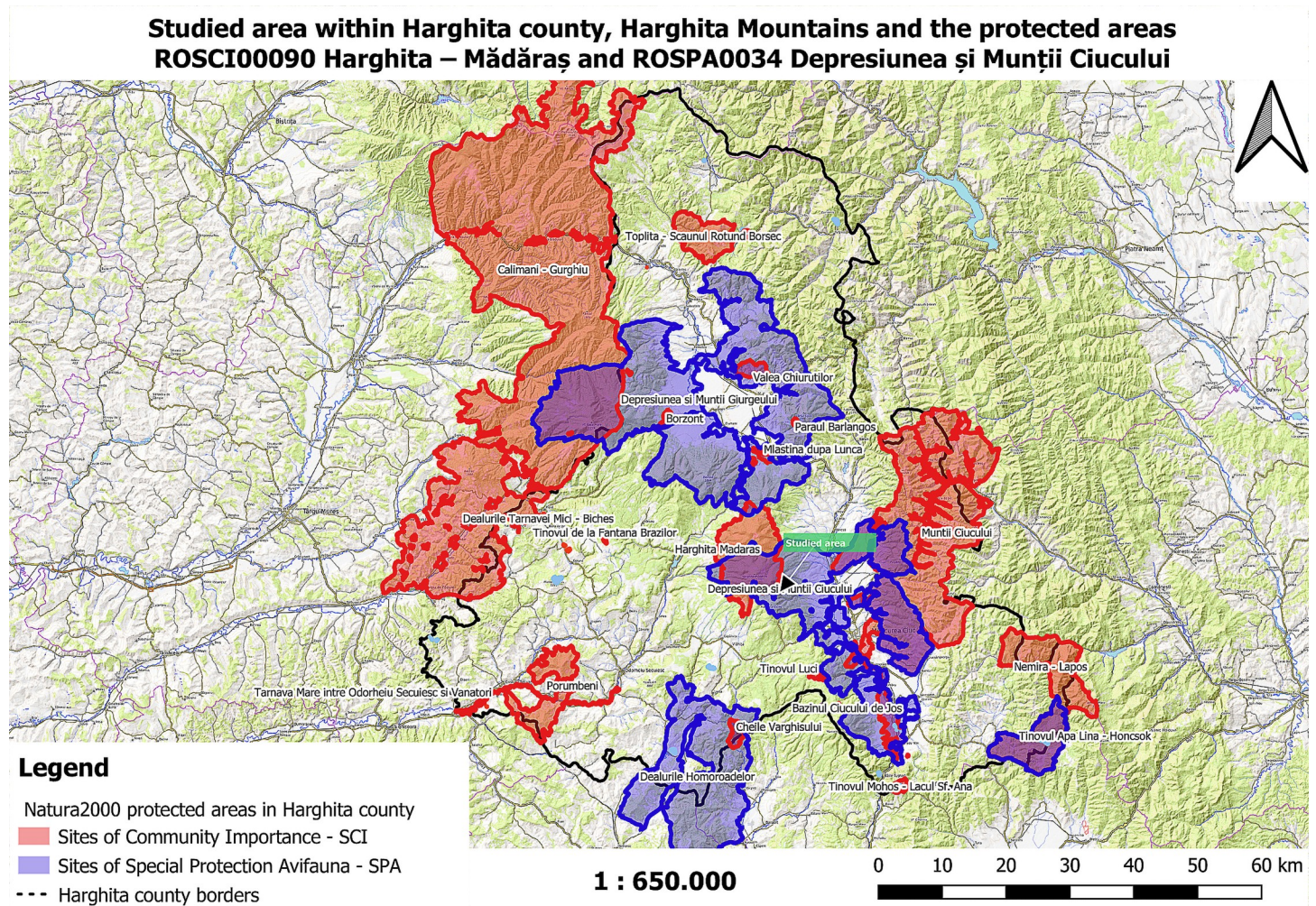


Fig 1. The map of the studied area within Harghita county, Harghita Mountains and the protected areas ROSCI00090 Harghita–Mădăraş and ROSPA0034 Depresiunea și Munții Ciucului (map used by permission of National Agency for Protected Areas Harghita Territorial Service, Romania).

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short, the nearest plants occurring within 1–2 meters, while others occurring within 10–15 meters from the hybrid.

P. albida subsp. *tricuspis* plants occurred in slightly higher numbers, within the drier parts of the swamp. The distances were somewhat longer, from 2 metres up to 10 metres or more (up to 40 meters), if we take in consideration the distance to the edges of the swamp, where scarce *P. albida* subsp. *tricuspis* plants were found.

We recommend it is imperious to put the entire studied area under strict protection since, shortly after the field studies were performed, the entire heath was completely destroyed by uncontrolled grazing and trampled by the hooves of hundreds of cattle that were walked over the heath.

Parental species description

***Dactylorhiza fuchsii* (Druce) Soó subsp. *sooana* (Borsos) Borsos, 1961.** Despite the fact that most of the area was carefully studied, we could not find any typical *D. fuchsii* (Druce) Soó (Common Spotted Orchid) plants, generally described as the tall, vigorous individuals, with

the specific deep purple-coloured, highly floriferous inflorescences. Instead, in the close proximity of the hybrid, we found one of its rarest and most elusive subspecies in Romania, *D. fuchsii* subsp. *sooana*.

Description. The habitus of the plants under study, fits perfectly the original description made in 1959, by the Hungarian botanist Olga Borsos (born 1926), where she described *D. fuchsii* subsp. *sooana* as endemic to the low mountain ranges of Hungary [16]. The Romanian plants were also compared to the typus/holotype registered in the *Flora Hungarica Herbarium* (Reg. No. 3328:152, Rev.: O. Borsos), at the Hungarian Natural History Museum, Bot. Dep. Budapest. *D. fuchsii* subsp. *sooana* is quite similar in habitus (physical aspect) to the type species, *D. fuchsii* (Druce) Soó, although it is slenderer and spindlier, with a slim, solid stem, 7–2 mm in diameter. The plants are on average shorter, reaching up to 30–40 centimetres in height, very rarely 50–60 centimetres (*D. fuchsii* plants are very sturdy, in some cases reaching up to 90–100 centimetres). The lowermost leaf (basal leaf) is elongated with an oval, roundish tip. It is distinguished from *D. maculata* (L.) Soó by the oval tip of the lowest leaf, the leaves being broadest in the upper half. The 5–8 cauline leaves are lanceolate to narrow-lanceolate, elongate, deep green, always brownish-purple spotted on the upper side (sometimes the spots are oval to flat-elongated in shape), wider around the middle/upper half. The inflorescence is elongated, lax to dense and floriferous and may bear up to 35–40 flowers. The flowers are medium-sized, pure white. In some rare cases, the lateral sepals and the spur are faintly tinged pink. The sepals are spreading obliquely to subtended angle of c.45° relative to the hood. The lateral petals and the dorsal sepal form a loose hood over the fertile anther (gynostemium). The labellum is white, three-lobed and spreading. The median lobe is prominent, triangular almost as broad as and longer than the lateral lobes, sometimes with a rounded tip. The markings on the labellum consist exclusively of few, short, irregular, purple-reddish dots, streaks, lines or irregular loops. One of the most important characteristics of this subspecies is that the purple markings never form continuous loops or circles. The lateral lobes are scalloped, flat, with unmarked lateral margins. The spur is slim, straight to horizontal, slightly curved downwards at tip. The spur secretes no nectar. The flower bracts are shorter than flowers, more or less equal to the ovary, except at the base of the inflorescence, where they are slightly longer than the ovaries, but shorter than the flowers (Figs 2–4).

Chromosome number. $2n = 40$ [similar to type species *D. fuchsii* (Druce) Soó]

Habitat. *D. fuchsii* subsp. *sooana* prefers swampy areas, very wet meadows, humid forest margins, on alkaline to slightly acidic substrates (in present study, it occurred on moist substrate of *Sphagnum* L. moss), up to 1700 meters altitude.

Distribution. In Romania, *D. fuchsii* subsp. *sooana* occurs exclusively within the protected area Harghita-Mădăraş, in medium homogenous populations [17]. Beside Hungary and Romania, *D. fuchsii* subsp. *sooana* was also mentioned to occur in the White Carpathians of Ukraine [18], Czech Republic and Slovakia [19].

Etymology. The generic name, *Dactylorhiza*, is a compound term that originates in the ancient Greek words *dáktylos* (finger) and *rhíza* (root), ad litteram meaning *finger-shaped* or *finger-like roots*, a reference to the *palmate, two- to five-lobed tubers* that resemble the *fingers of a human palm*, a characteristic of all the species belonging to this genus.

The specific epithet, *fuchsii*, was given in honour of Leonhart/Leonhard Fuchs (1501–1566), a German physician and botanist, hence its common name, Fuchs' *Dactylorhiza*. Taxa with the specific epithet, *fuchsii*, commemorate his name. Due to the strongly *maculated leaves*, this species is also known by its vernacular names, the Common Spotted Orchid, the Spotted Orchid or the Marsh Spotted Orchid.

The *infraspecific epithet (subspecies epithet)*, *sooana*, was given in honour of Károly Rezső Soó von Bere (1903–1980), a Hungarian botanist, professor at the University of Budapest,



Fig 2. *Dactylorhiza fuchsii* (Druce) Soó subsp. *sooana* (Borsos) Borsos entire plant in its natural habitat (Fig 2), details of the inflorescences (Figs 3 and 4). One of the inflorescences (Fig 4) is visited by hoverfly of the family Syrphidae Latreille, 1802. The hoverflies, which are true bee-mimics, are very frequent visitors of *D. fuchsii* subsp. *sooana*, although it has not been shown to be its real pollinators, as of yet. Figs 2–4 © 2020 N. Anghelescu originals.

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born in Odorheiu Secuiesc, Romania, hence its vernacular name, Soó's Spotted-orchid. Taxa with the specific epithet, *sooana*, commemorate his name.

***Pseudorchis albida* (L.) Á.Löve & D.Löve subsp. *tricuspis* (Beck) E.Klein, 2000.** *Description.* *P. albida* subsp. *tricuspis* is a slender, tall plant that may reach 20–35 (occasionally 40–45) centimetres in height. The stem is erect, greenish, 3.4–3.9 millimetres in diameter. The basal leaf is widest around the middle. The 5–8 cauline leaves are shiny-green to yellowish-green, oblong-obovate, widest around the middle, briefly pointed. The cauline leaves (stem leaves) are narrower, lanceolate, the upper ones becoming, pointed, bract-like. The inflorescence is up



Fig 3. *Dactylorhiza fuchsii* (Druce) Soó subsp. *sooana* (Borsos) Borsos entire plant in its natural habitat (Fig 2), details of the inflorescences (Figs 3 and 4). One of the inflorescences (Fig 4) is visited by hoverfly of the family Syrphidae Latreille, 1802. The hoverflies, which are true bee-mimics, are very frequent visitors of *D. fuchsii* subsp. *sooana*, although it has not been shown to be its real pollinators, as of yet. Figs 2–4 © 2020 N. Anghelescu originals.

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to 6–9 centimetres long, dense, narrow-cylindrical, very floriferous, bearing up to 35–65 small, yellowish-green flowers. The flowers are small, whitish to yellowish-green with complete or partial resupination [20]. The lateral petals and sepals are very similar and form a tight hood over the anther/gynostemium. The labellum is the main distinctive feature of the species. It is yellow, flat, deeply three-lobed with all three lobes of equal lengths. Spur is 2.5–3.4 millimetres long, cylindrical, slightly curved downwards, yellowish-white, blunt and secretes abundant nectar. The flower bracts of the lower/basal flowers of the inflorescence are narrow-lanceolate, longer than the top ones (Figs 5–7). In general, bracts are longer than the ovaries, but shorter than the flowers [21].

Chromosome number. $2n = 42$ [22].

Habitat. In Romania it grows in well-drained to wet meadows, grasslands, pastures, forest edges and rocky slopes, alpine pastures and meadows, at 700–2300 meters altitude. It prefers a wide range of soil conditions from the acidic moist substrata of *Sphagnum* bogs to alkaline/calcareous, well drained soils on carboniferous limestone [23]. Plants having attributes of *P. albida* subsp. *tricusps* grow on acidic soils in the Czech Republic [24].



Fig 4. *Dactylorhiza fuchsii* (Druce) Soó subsp. *sooana* (Borsos) Borsos entire plant in its natural habitat (Fig 2), details of the inflorescences (Figs 3 and 4). One of the inflorescences (Fig 4) is visited by hoverfly of the family Syrphidae Latreille, 1802. The hoverflies, which are true bee-mimics, are very frequent visitors of *D. fuchsii* subsp. *sooana*, although it has not been shown to be its real pollinators, as of yet. Figs 2–4 © 2020 N. Anghelescu originals.

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Distribution. *P. albida* subsp. *tricuspis* is a palearctic species, covering boreal alpine, subalpine and temperate zones, from Europe to the Russian Far East, to the Northern Urals and Kamchatka and from Eastern Canada to Greenland, but not in Siberia.

Etymology. The Catholic priest and renowned Italian botanist Pier Antonio Micheli (1679–1737) was the first to illustrate and use the name *Pseud-orchis* (*false orchid*) for this genus, in his work *Nova Plantarum Genera* (1729), in order to indicate the similar appearance of the plant to the members of the genus *Orchis*.

The generic name, *Pseudorchis*, originates in the ancient Greek words *pseudos* (false) and *orkhis* (*testicle* or *a plant with roots like testicles*), a word used for the first time by Theophrastus (372–286 B.C.E.), in his book *Historia Plantarum* (*Enquiry into Plants*). Consequently, *Pseudorchis* may be interpreted as the *false-orchid*, which refers to the shape of its *root-tubers* that are not *round*, like all the other *Orchis* species, but *bifid* or *palmate*, hence the vernacular name of these species, the False Orchid.

The specific epithet, *albida*, has its origin in the Old Latin word *alba*, which means white and refers to the *whitish flowers* of this subspecies, hence its vernacular names, the Small White Orchid, the White Mountain Orchid or the Bright White Pseudorchis.

The subspecific (infraspecific) epithet, *tricuspis*, originates in the ancient Greek words *tria* (three) and *cuspis* (tip) meaning *with three tips*, a reference to the three prominent lobes of the



Fig 5. *Pseudorchiza albida* (L.) Á.Löve & D.Löve subsp. *tricuspis* (Beck) E.Klein entire plant in its natural habitat (Fig 5), details of the inflorescences (Figs 6 and 7). One of the inflorescences (Fig 7) is visited by hoverfly of the family Syrphidae Latreille, 1802. The hoverflies, which are true bee-mimics, are also very frequent visitors of *Pseudorchiza albida* subsp. *tricuspis*, although it has not been shown to be its real pollinators, as of yet. Figs 5–7 © 2019 N. Angelescu originals.

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labellum, hence its potential vernacular names, the Small White Trilobate Orchid or the Trilobate White Pseudorchis.

Flowering times

The flowering times of parental species and the resulted hybrid overlapped entirely, from mid-June to late July. At the time when the studies were performed, all three species were at the peak of anthesis (flowering times). In some parts of the swamp, some *D. fuchsii* subsp. *sooana* individuals were slightly off the peak of anthesis, which indicates that *D. fuchsii* subsp. *sooana* parent might have an earlier blooming time, with approximately 3–5 days before *P. albida* subsp. *tricuspis* parent. Nevertheless, this slight synchronicity mismatch is insignificant and does not influence the frequency of cross-pollination events, which might potentially result in the occurrence of the hybrid.



Fig 6. *Pseudorchis albida* (L.) Á.Löve & D.Löve subsp. *tricuspis* (Beck) E.Klein entire plant in its natural habitat (Fig 5), details of the inflorescences (Figs 6 and 7). One of the inflorescences (Fig 7) is visited by hoverfly of the family Syrphidae Latreille, 1802. The hoverflies, which are true bee-mimics, are also very frequent visitors of *Pseudorchis albida* subsp. *tricuspis*, although it has not been shown to be its real pollinators, as of yet. Figs 5–7 © 2019 N. Angelescu originals.

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Nomenclature

× *Pseudorhiza nieschalkii* (Senghas) P.F.Hunt nothosubsp. *sicolorum* H.Kertész & N.Angelescu, 2020, nothosp. Nov. The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix <http://ipni.org/>. The online version of this work is archived and available from the following digital repositories: PubMed Central and LOCKSS.

General descriptions of the hybrid × *Pseudorhiza nieschalkii* (Senghas) P.F. Hunt nothosubsp. *sicolorum* H.Kertész & N.Angelescu and parental species comparison diagrams

In general, primary hybrids (F₁ generation) appear phenotypically intermediate between the parental species (Fig 8).

While researching the existing orchid species of the heathland, she spotted an oddly looking plant, which, from a distance, resembled *P. albida* subsp. *tricuspis*. She approached the plant and, after parting the dense grass that surrounded it, she realised it was something more than a simple *P. albida* subsp. *tricuspis*. Minutes later digital images were sent to N. Angelescu who confirmed the putative parents and, in that regards, suggested that the hybrid may actually be new to science. Photo © 2020 H. Kertész original

Nevertheless, in the case of × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum*, a rather more notable influence of *P. albida* subsp. *tricuspis* parent is evident (Figs 9–11).

Morphometric/biometric methods

Given that the parental species differ considerably in morphology, the identification of any hybrids between them, appear relatively straight-forward (the images are very explicit). In

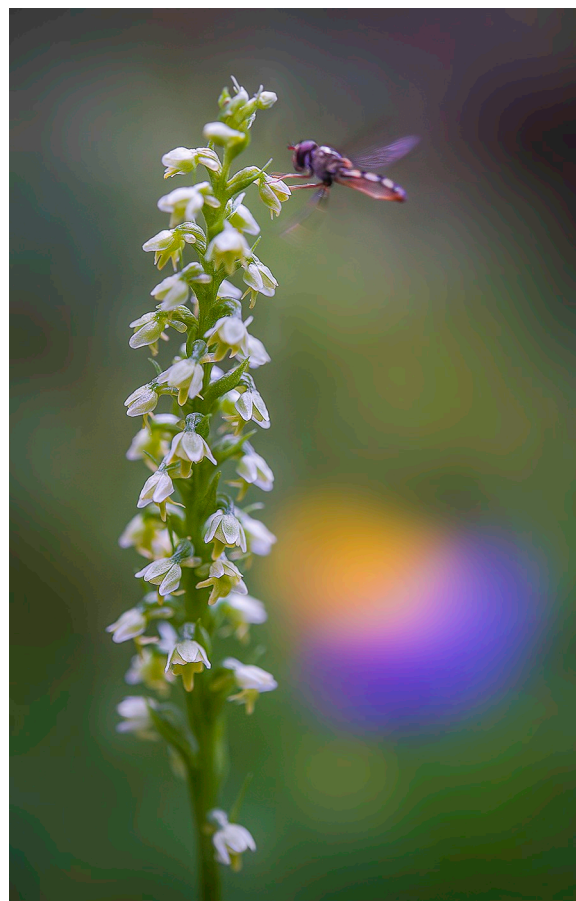


Fig 7. *Pseudorchis albida* (L.) Á.Löve & D.Löve subsp. *tricuspis* (Beck) E.Klein entire plant in its natural habitat (Fig 5), details of the inflorescences (Figs 6 and 7). One of the inflorescences (Fig 7) is visited by hoverfly of the family Syrphidae Latreille, 1802. The hoverflies, which are true bee-mimics, are also very frequent visitors of *Pseudorchis albida* subsp. *tricuspis*, although it has not been shown to be its real pollinators, as of yet. Figs 5–7 © 2019 N. Angelescu originals.

<https://doi.org/10.1371/journal.pone.0241733.g007>



Fig 8. This is the first image of × *Pseudorhiza nieschalkii* nothosubsp. *siculorum* ever taken by H. Kertész at 08:39:19am, on 30th of June, 2020.

<https://doi.org/10.1371/journal.pone.0241733.g008>

most cases, the phenotypical characters/traits of the hybrid appear to resemble more *P. albida* subsp. *tricuspis* parent.

It should be emphasised that a positive determination of hybrids implies a deep knowledge of the variation of the parental species and a deep characterisation of the biotope in which they are found. Therefore, for the correct identification of a hybrid, it is imperious that at least one unequivocal character of each parental partner is demonstrable and cannot come from the other supposed partner [25].

In order to describe this unique hybrid as comprehensive as possible, a wide range of characters were taken in consideration and biometrically/morphometrically analysed. All



Fig 9. Morphological comparisons of × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* and parents. The following three comparison diagrams show the intermediate characters of the hybrid's habitus (Fig 9), inflorescence (Fig 10) and flowers (Fig 11), compared to those of its parents. The hybrids are almost as tall as the parents. The hybrid's inflorescence is less floriferous and flat topped, resembling more that of *D. fuchsii* subsp. *soana* parent. The flowers' labellum is deeply three-lobed, very similar to *P. albida* subsp. *tricuspis*' labella. Figs 9–11 © 2020 N. Angelescu originals.

<https://doi.org/10.1371/journal.pone.0241733.g009>

morphological measurements were undertaken in the field. In total, 68 morphological characters were measured directly. Morphological characters used for analysis included most of the characters used previously in [26–28]. Special attention was given to the characters that proved to be taxonomically informative and those that involve the details of labellum morphology. The quantitative measurements encompass all organs except the root-tubers and the reproductive organs (fused forming the gynostemium). Measurements are examples of the parental plants and of the hybrid (Table 1).

Chromosome counts

Explants, 3–4 millimetres long adventitious roots tips (meristematic root tips), were sampled from the hybrid and its parents. Chromosome counts were carried out using preparations of



Fig 10. Morphological comparisons of × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* and parents. The following three comparison diagrams show the intermediate characters of the hybrid's habitus (Fig 9), inflorescence (Fig 10) and flowers (Fig 11), compared to those of its parents. The hybrids are almost as tall as the parents. The hybrid's inflorescence is less floriferous and flat topped, resembling more that of *D. fuchsii* subsp. *sooana* parent. The flowers' labellum is deeply three-lobed, very similar to *P. albida* subsp. *tricuspis*' labella. Figs 9–11 © 2020 N. Anghelescu originals.

<https://doi.org/10.1371/journal.pone.0241733.g010>

dividing cells (at metaphase) from the apical portion of adventitious roots. The root tips were pre-treated in a 0.5% colchicine and incubated for 2 h at 10–15°C. These were transferred to Clark fixative 3:1 (v/v) (3 parts absolute ethanol and 1 part glacial acetic acid) at 10–15°C for 30 min and then 3–4°C for 48 h. Further, meristematic root tips were hydrolysed at 60°C in 1 N HCl for 60 min and stained in Feulgen stain. For cytogenetic analysis, the stained root tips were macerated on a microscope slide in a drop of acetocarmine for 1–2 min, covered with a coverslip and squashed manually (classical squash method in acetocarmine).

Results

Morphometric comparisons

The morphometric results proved to be partially asymmetric. The most effective diagnostic characters, such as labellum morphology, placed the hybrid closer to the *P. albida* subsp. *tricuspis* parent, rather than *D. fuchsii* subsp. *sooana* parent.

Habitus. The hybrid was tall and sturdy, reaching up to 28.5 centimetres, intermediate or even similar in size to the parents, which both usually range between 25–40 centimetres in



Fig 11. Morphological comparisons of × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* and parents. The following three comparison diagrams show the intermediate characters of the hybrid's habitus (Fig 9), inflorescence (Fig 10) and flowers (Fig 11), compared to those of its parents. The hybrids are almost as tall as the parents. The hybrid's inflorescence is less floriferous and flat topped, resembling more that of *D. fuchsii* subsp. *sooana* parent. The flowers' labellum is deeply three-lobed, very similar to *P. albida* subsp. *tricuspis*' labella. Figs 9–11 © 2020 N. Angelescu originals.

<https://doi.org/10.1371/journal.pone.0241733.g011>

height (taller parental species that may reach up to 40–60 centimetres are very rare and were not present among the individuals included in this study).

Stem. The stem is slender and slim, vividly-green coloured. It presents no significant purple pigmentation at the tip (only trace amounts of anthocyanins), resembling exclusively *P. albida* subsp. *tricuspis* parent.

Leaves. The hybrid presents 1 unspotted lanceolate basal leaf and 4 narrow-lanceolate cauline leaves (stem leaves), the upper one being very reduced, bract-like, not reaching the inflorescence. The leaves present no maculae or any other pigmentation, a character inherited exclusively from *P. albida* subsp. *tricuspis* parent that has unspotted, bright-green leaves, therefore, the inheritance of leaf characters proved to be asymmetrical.

Inflorescence. The inflorescence is not as floriferous as *P. albida* subsp. *tricuspis* parent. It resembles mostly *D. fuchsii* subsp. *sooana* parent, both in shape—cylindrical, with a flat top, and number of flowers—it is less floriferous, bearing approximately 28 medium sized flowers.

Flowers. The flowers' size of the hybrid are intermediate between the parents and range mostly in the median range of the parental sizes (length × width), being slightly larger than those of *P. albida* subsp. *tricuspis* parent and smaller to almost equal in size to *D. fuchsii* subsp. *sooana* parent.

Bracts. The flower bracts are slightly variable in size, the lower bracts being longer than the flowers, while the top bracts being slightly longer than the ovaries but shorter than the flowers. Overall the bracts resemble mostly *P. albida* subsp. *tricuspis* parent, since *D. fuchsii* subsp. *sooana* parent has bracts shorter to almost equal to the ovaries. The top flower bracts show a faint purple pigmentation, a trait mildly inherited exclusively from *D. fuchsii* subsp. *sooana* parent and totally absent from *P. albida* subsp. *tricuspis* parent.

Sepals & lateral petals. The sizes are again, intermediary between the parental species, but the colour and pinkish markings are mainly inherited from *D. fuchsii* subsp. *sooana* parent.

Table 1. Morphometric comparison of the putative parental species and the unique resulted hybrid.

Vegetative & Floral Organs	Characters / Features (millimetres)	<i>Dactylorhiza fuchsii</i> subsp. <i>sooana</i>	× <i>Pseudorhiza nieschalkii</i> nothosubsp. <i>sicolorum</i>	<i>Pseudorchis albida</i> subsp. <i>tricuspis</i>
1. Stem & Inflorescence				
	Overall height	333/400	285	300/350
	Stem diameter	3.5/3.7	3.2	3.4/3.9
	Stem anthocyanins	moderate/strong	traces apically	absent
	Inflorescence length	53/60	67 (23% of stem length)	60/80 (90)
	Inflorescence shape	conical 25/40	cylindrical, flat topped	elongated, cylindrical, acuminate top
	No. of flowers	25/40	28	45/65
2. Leaves				
	No. of basal leaves	1, rounded at tip	1, widest around the middle	1, widest around the middle
	Distribution of sheathing leaves on stem	Even/lower half	basal/even	denser basally/even
	Longest leaf posture	Spreading, to subtend angle of c.45° relative to the stem	spreading horizontally to subtend angle of c.90° relative to the stem	Spreading, to subtend angle of c.45° (90° in some cases) relative to the stem
	No. of sheathing leaves	05-Aug	4	05-Aug
	No. of non-sheathing leaves	1	1	-
	Length of longest leaf	70/74	65	22/72
	Width of longest leaf	12/13.2	11	Nov-15
	Outline shape of longest leaf	linear-lanceolate, broadest in the upper half	linear-lanceolate	linear-lanceolate, widest around the middle
	Leaf conduplicate	strong	faint/absent	strong
	Apex hooding	moderate/faint	lacking pronounced apical hooding	moderate/strong
	Leaf colour	deep green	vivid/shiny green	shiny, light to deep green
	Leaf dorsal side	deep green	green	deep green
	Leaf ventral side	green	green	deep green, veined
	Leaf margins	entire	entire	entire
	Leaf markings	brownish-purple spotted on the upper side	unmarked	unmarked
	Upper leaves	shorter, narrow-lanceolate, acuminate	bract-like	shorter, narrow-lanceolate, acuminate, bract-like
3. Bracts & Ovary				
	Length of basal bracts	8/13 exceeding the ovaries	14/16, exceeding flowers	10/16, exceeding the flowers
	Width of basal bracts	1.5/2.3	1.9/2.5	2.8/3.2
	Length of floral bracts	11/8, equal/slightly longer than the ovaries	13/10, longer/equal to the ovaries	12/9, longer than the ovaries
	Width of floral bracts	1.2/2.1	1.1/1.4	2.1/3
	Texture of bracts	membranous/robust	robust/membranous	robust
	Bract anthocyanins	strong	traces present in the top bracts	absent
	Marginal wall thickness	thin	moderate	thick
	Ovary length	5.9/7.3	5.2/7	4.3/5.7
	Ovary diameter	1.2/2.2	1.2/1.6	2.1/3.2
	Ovary anthocyanins	strong	traces	absent
4. Sepals & Lateral Petals				
	Lateral sepals position	spreading to subtend angle of c.45° relative to the hood	spread horizontally, laterally relative to the hood	tightly connivent forming the hood

(Continued)

Table 1. (Continued)

Vegetative & Floral Organs	Characters / Features (millimetres)	<i>Dactylorhiza fuchsii</i> subsp. <i>sooana</i>	× <i>Pseudorhiza nieschalkii</i> nothosubsp. <i>sicolorum</i>	<i>Pseudorchis albida</i> subsp. <i>tricuspis</i>
	Lateral sepals connivant	no	no	yes
	Sepal fusion from base (%)	-	05-Oct	90/95
	Sepal apex	acuminated	oval/blunt	oval/roundish
	Sepal iridescent green pigment	absent	absent	present
	Median sepal length	5.2/6/7	4.8/5.7	4.3/5.8
	Median sepal width	3.2/4.6	2.7/3.7	2.4/3.8
	Lateral sepals width	1.7/2.4	2.1/2.9	2.5/3.9
	Lateral sepals length	4.6/5.7	4.7/5.8	3.9/4.5
	Lateral petals width	2.4/3.2	1.7/2.3	2.4/3.7
	Lateral petals length	3.9/4.8	3.8/4.6	3.8/4.7
5. Labellum				
	Outline shape	orbicular, nearly flat	orbicular, deeply three-lobed, nearly flat	deeply three-lobed
	Lobes	Large, scalloped	three, triangular-elongated, equal in length	three, thin-elongated, equal in length
	Median lobe length	2.1/2.3	2.9/3.1	2.3/3.4
	Lateral lobes length	1.4/1.9	2.6/2.9	2.1/3.3
	Sinuses separating the three lobes	shallow	very deep, c.2.1/2.3	very deep, c.2.2/2.4
	Width	3.9/4.8	1.4/2.3	0.9/1.2
	Lateral lobe reflexion	slightly reflexed upwards	moderate to slightly recurved backwards	moderate to slightly recurved backwards
	Central lobe apex	shallow, oval-roundish	acuminate, entire, flat	acuminate-pointy, entire, flat
	Central lobe width	4.2/5.3	1.8/2.1	1.6/2
	Base colour of labellum	white	white with a yellowish splash at spur entrance	yellowish-green
	Centre colour	white, marked with scattered dots and streaks that never form circular loops	whitish, convex, uniformly dotted	yellowish, unmarked
	Margins colour	white	white	yellowish
	Markings type	purple dots and streaks that never form circular loops	scattered faint pinkish dots & streaks, never forming circular loops	uniformly yellow, unmarked
	Surface markings papillate	moderately	moderately	no
	Markings distribution in centre	Centrally, lateral sides of the lobes white	uniformly scattered	circular
	Markings contrast	strong	weak	-
	Lateral lobe indentation	entire, scalloped	entire/moderate indentation, scalloped, slightly recurved	entire
6. Spur				
	Spur length	3.2/4/5	3.2/3.8	2.5/3.4
	Spur shape	cylindrical thin, elongated, shorter than the ovary	cylindrical, thin, with a roundish tip, c. ½ ovary length	Cylindrical, blunt, whitish, c. ½ ovary length
	Spur width entrance	2.2/2.8	1.3/2	1.1/2
	Spur width halfway	1.4/1.7	1.2	1.1/1.2
	Spur down-curvature	straight to slightly down-curved	slightly down-curved	slightly down-curved
7. Nectar				

(Continued)

Table 1. (Continued)

Vegetative & Floral Organs	Characters / Features (millimetres)	<i>Dactylorhiza fuchsii</i> subsp. <i>sooana</i>	× <i>Pseudorhiza nieschalkii</i> nothosubsp. <i>sicolorum</i>	<i>Pseudorchis albida</i> subsp. <i>tricuspis</i>
	Presence	no	-	yes
	Amount	-	-	abundant
8. Smell				
	Fragrance type	vaguely fruity	-	moderate/strong, sweet, vanilla like

The quantitative measurements (in millimetres unless otherwise stated) encompass all organs except the root-tubers and gynostemium.

<https://doi.org/10.1371/journal.pone.0241733.t001>

The background colour of the tepals is white with faint purple-pink dots and streaks on the lateral sepals, a characteristic exclusively inherited from *D. fuchsii* subsp. *sooana* parent, with the mention that hybrid's pigmentation is less pronounced, the anthocyanins being expressed in far smaller amounts.

Helmet/hood. The lateral petals and sepals that construct the helmet, which protects and covers the reproductive organs (fused to form the gynostemium) are not so tightly folded. The lateral sepals significantly resemble *D. fuchsii* subsp. *sooana* parent as they are laterally/horizontally spreading, hence the loose helmet is formed mainly by the dorsal sepal and lateral petals, completely different from the tight helmet formed from the highly connivent sepals and lateral petals in *P. albida* subsp. *tricuspis* parent.

Labellum. The morphology of the labellum is particularly interesting as the parents differ considerably in the labellum size and shape. The overall shape of the labellum closely resembles *P. albida* subsp. *tricuspis* parent. The labellum is deeply three-lobed with all lobes almost equal in length and width, a characteristic exclusively inherited from *P. albida* subsp. *tricuspis* parent, although they are wider than those of *P. albida* subsp. *tricuspis* parent, a feature partly inherited from *D. fuchsii* subsp. *sooana* parent, which has scalloped, rounded labellar lobes.

Labellar colour and markings. Labellum colour is intermediate between the parents. The yellowish background colour of the labellum base (spur entrance) is inherited from *P. albida* subsp. *tricuspis* parent, which has yellowish-green flowers. The white background colour of the labellum and the faint pinkish marks represent the distinctive features of this nothosubspecies, clearly inherited from *D. fuchsii* subsp. *sooana* parent. The pinkish markings are distinctively fainter but resemble *D. fuchsii* subsp. *sooana*'s patterns of scattered dots and streaks that never form circular loops.

Spur. The spur mostly resembles *D. fuchsii* subsp. *sooana* parent, being slightly longer and slenderer than in *P. albida* subsp. *tricuspis* parent (but shorter than the ovary), cylindrical and slightly curved downwards.

Chromosome counts

Stained cells were viewed using Micros Microscope, 100X lenses and photographed with Optika microscopes camera, lenses 0,45X. Images were analysed using OptikaSview, developed by OPTIKA Microscopes Software (Figs 12–14).

Chromosomes were counted for the hybrid and confirmed expected numbers: × *P. nieschalkii* nothosubsp. *sicolorum* $2n = 40 (\pm 2)$, which makes the intergeneric hybrid a diploid (allopolyploid) nothosubspecies. These findings also comply with the fact that the parental species have close values of the chromosome number, $2n = 40$ & 42 , allowing them to cross-pollinate relatively easily (Table 2).

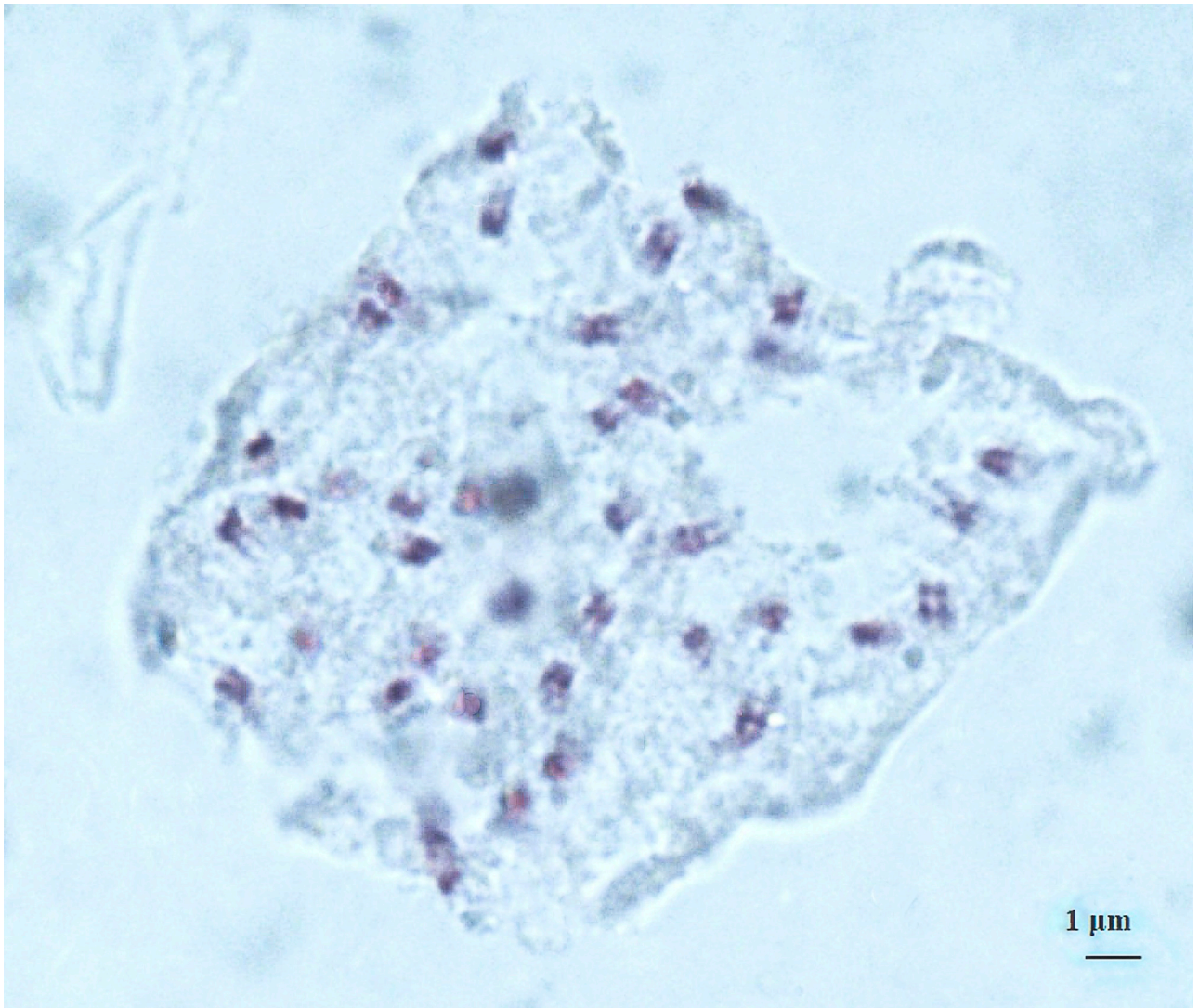


Fig 12. Chromosomes at somatic metaphase (Feulgen staining) of × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* hybrid $2n = 40 (\pm 2)$, no. 28–2 (Fig 12); no. 30–1 (Fig 13); no. 31–4 (Fig 14). Scale bar = 1 μ m.

<https://doi.org/10.1371/journal.pone.0241733.g012>

Description

× *Pseudorhiza nieschalkii* (Senghas) P.F.Hunt nothosubsp. *sicolorum* Kertész & Anghel. 77216534–1

Hybrid formula. *Dactylorhiza fuchsii* (Druce) Soó subsp. *sooana* (Borsos) Borsos × *Pseudorchis albida* (L.) Á.Löve & D.Löve subsp. *tricuspis* (Beck) E. Klein

Diagnosis. Morphologically intermediary between the parents. Stem and leaves similar to *P. albida* subsp. *tricuspis* by the vividly green colour and the absence of spots on the leaves. The influence of *D. fuchsii* subsp. *sooana* parent may be seen in the whitish colour of the flowers and faint pinkish marks on the lateral sepals and the labellum. The marks are significantly fainter and resemble the scattered dots and lines found on *D. fuchsii* subsp. *sooana* 's labellum, which never form circular loops. The most distinctive feature is the three-lobed labellum, with all three lobes of equal length and width, most similar to *P. albida* subsp. *tricuspis* parent.

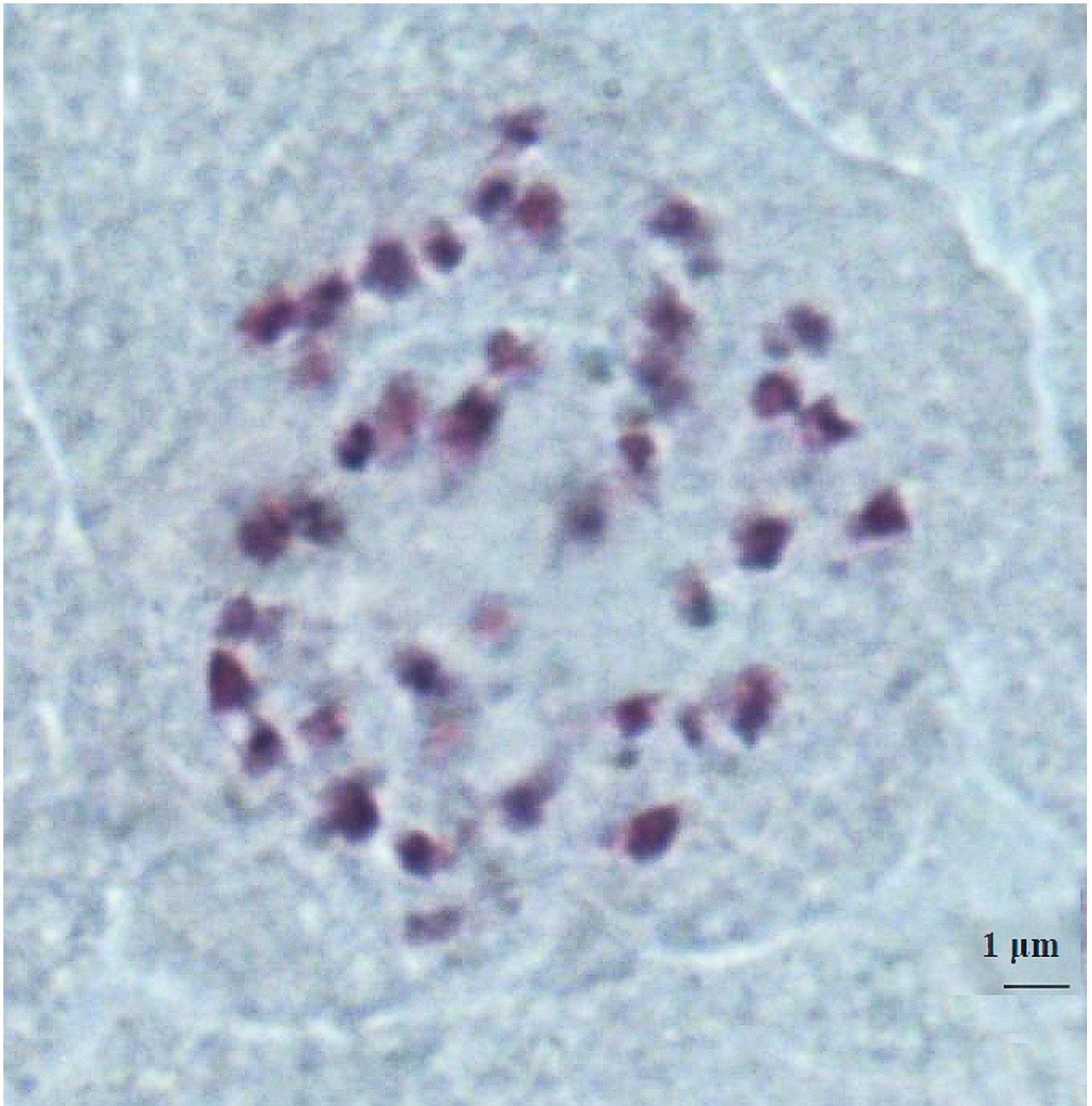


Fig 13. Chromosomes at somatic metaphase (Feulgen staining) of × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* hybrid $2n = 40 (\pm 2)$, no. 28–2 (Fig 12); no. 30–1 (Fig 13); no. 31–4 (Fig 14). Scale bar = 1 μm .

<https://doi.org/10.1371/journal.pone.0241733.g013>

Description. Stem 28.5 centimetres, 3.2 millimetres in diameter, devoid of anthocyanins. Basal sheath present. Sheathing leaves 4, evenly distributed evenly on the stem, angled, laterally spreading, narrowly lanceolate, longest leaf 65 millimetres long, widest leaf 11 millimetres wide, longest placed basally/base of the stem. Leaves vivid green, unmarked, lacking pronounced apical hooding. Inflorescence 67 millimetres (23% of stem length), cylindrical, flat topped, fairly lax. Flowers 28. Labellum deeply three-lobed, width × length 5.1 × 4.2 millimetres to central lobe, 3.9 millimetres to lateral lobes. Inter-lobe sinuses deep, c.2.1–2.3

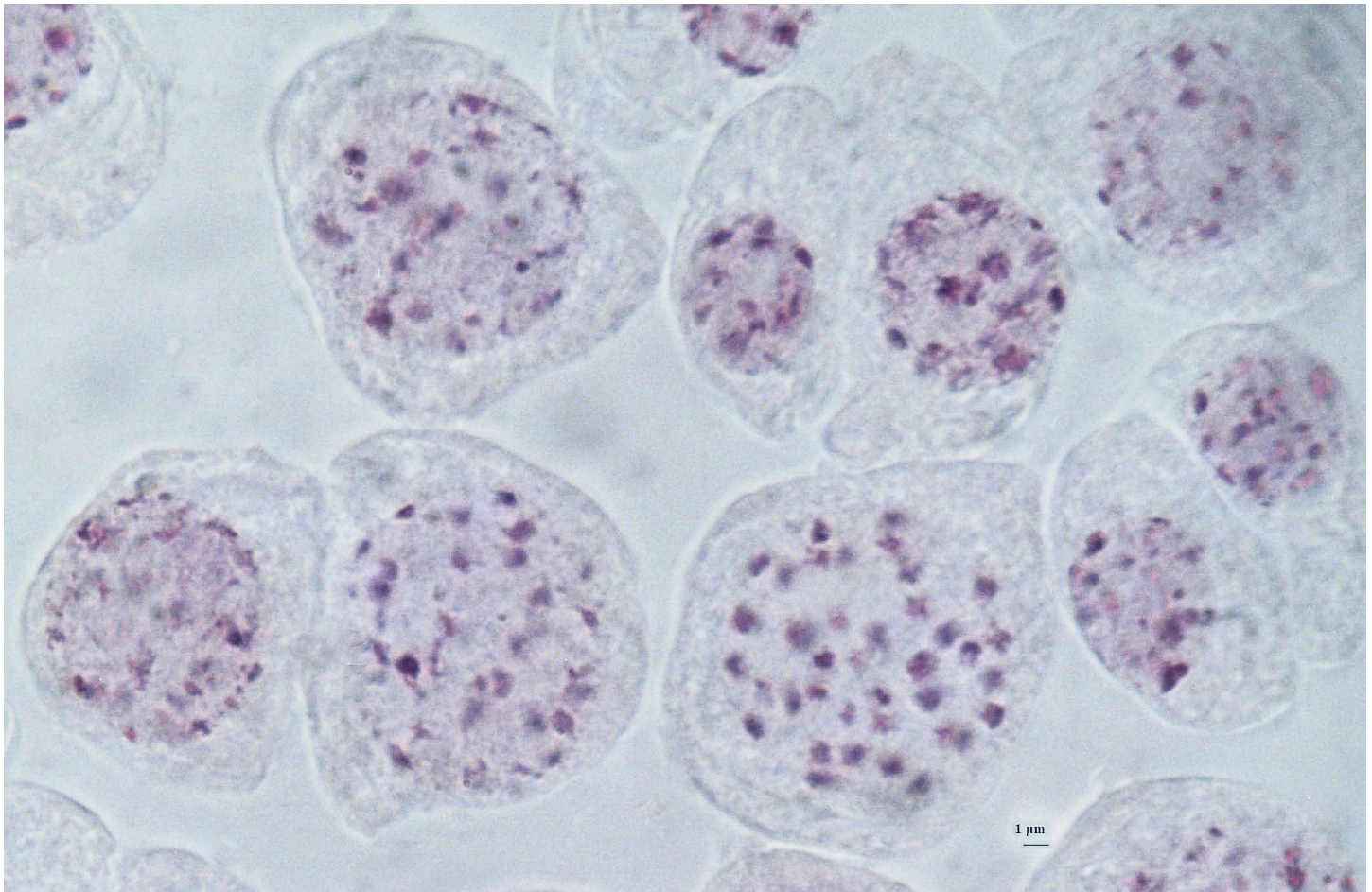


Fig 14. Chromosomes at somatic metaphase (Feulgen staining) of × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* hybrid $2n = 40 (\pm 2)$, no. 28–2 (Fig 12); no. 30–1 (Fig 13); no. 31–4 (Fig 14). Scale bar = 1 μm .

<https://doi.org/10.1371/journal.pone.0241733.g014>

millimetres deep. Lateral lobes only slightly recurved, presenting partial margin crenulation. Labellar base colour yellow with a greenish tinge at spur base, while labellar surface became gradually pure white. Markings comparatively low-contrast pink dots and streaks, covering the central region of labellum and the three lobes. Markings never form continuous loops. Labellar spur cylindrical, 3.4 millimetres long × 1.5 millimetres wide at mouth, 1.2 millimetres midway along length, slightly down-curved, shorter than the ovary. Lateral sepals lightly

Table 2. Chromosome numbers of the specimens studied.

Taxon	Distance from hybrid (m)	Chromosome no.
<i>D. fuchsii</i> subsp. <i>sooana</i>	2m	$2n = 40$
<i>P. albida</i> subsp. <i>tricuspis</i>	7m	$2n = 42$
× <i>P. nieschalkii</i> nothosubsp. <i>sicolorum</i> no. 28–2	-	$2n = 40 (\pm 2)$
× <i>P. nieschalkii</i> nothosubsp. <i>sicolorum</i> no. 30–1	-	$2n = 40 (\pm 2)$
× <i>P. nieschalkii</i> nothosubsp. <i>sicolorum</i> no. 31–4.	-	$2n = 40 (\pm 2)$

We chose to officially describe this unique hybrid between *D. fuchsii* subsp. *sooana* and *P. albida* subsp. *tricuspis*, as an **intergeneric hybrid, new to science**.

<https://doi.org/10.1371/journal.pone.0241733.t002>

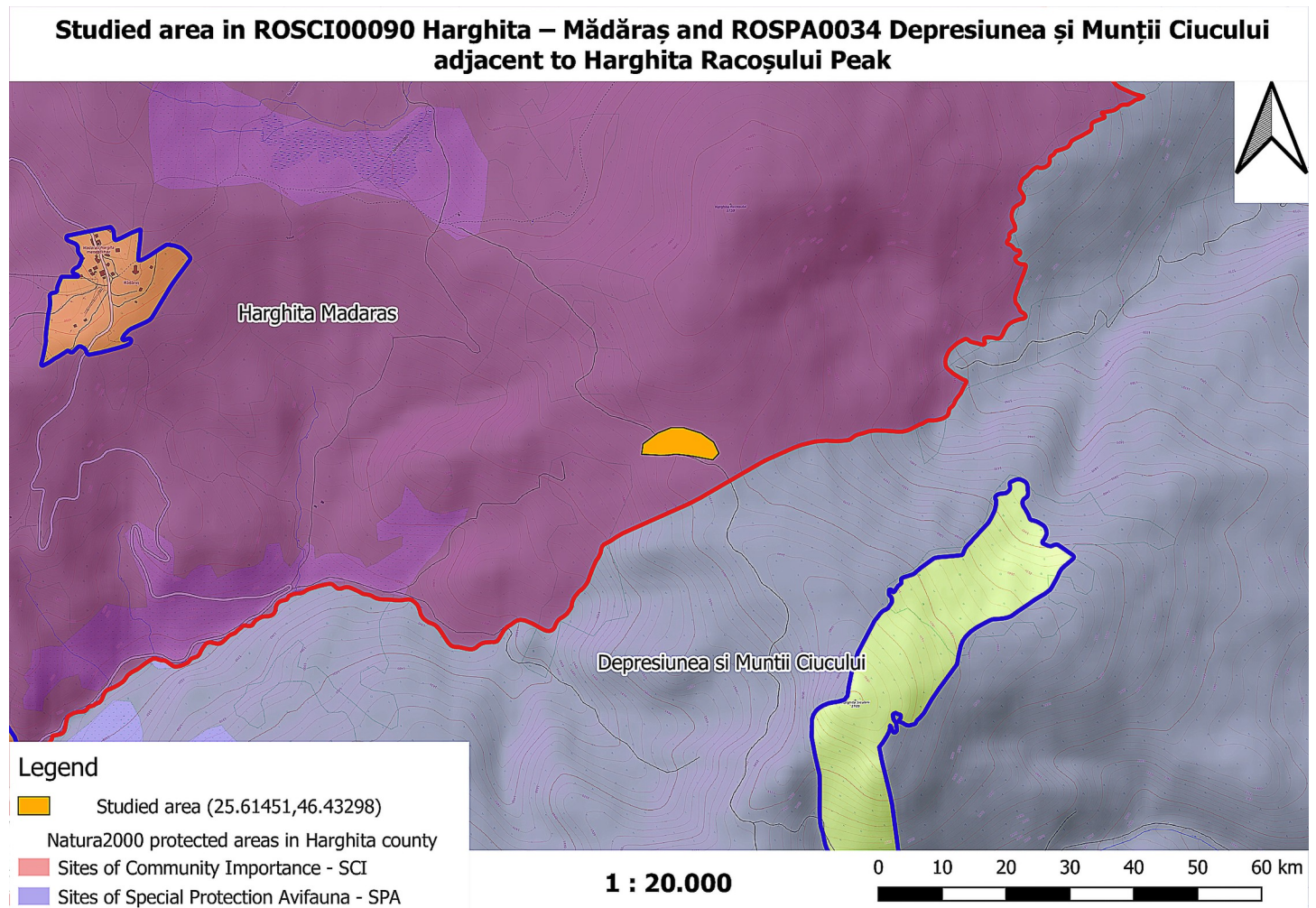


Fig 15. The distribution map of × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* in Harghita–Mădăraş protected area that yields the holotype and only known plant (map used by permission of National Agency for Protected Areas Harghita Territorial Service).

<https://doi.org/10.1371/journal.pone.0241733.g015>

sprinkled with purple dots and streaks, spreading laterally and horizontally, relative to the loose hood formed by the median sepal and lateral petals. Basal bracts 16 millimetres, exceeding flowers, upper floral bracts 14 millimetres, both exceeding ovaries, top bracts lightly purple tinged with anthocyanin pigments.

Chromosome number. $2n = 40$.

Flowering time. From mid-June to mid-July.

Locus classicus. × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* is the only known plant, an F_1 intergeneric hybrid, found on 30 June 2020, within the Natura 2000 protected area ROSCI00090 Harghita–Mădăraş and ROSPA0034 Depresiunea și Munții Ciucului, in the peat bog area adjacent to Harghita Racoşului Peak, next to the mountain refuge, up to 1,600–1,700 meters altitude. GPS: 25.61451, 46.43298, currently in Harghita County Romania (Fig 15).

Habitat. × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* prefers a sunny, swampy meadow, next to mixed forest margin, on mildly acidic, nutrient poor substrate. The area under study was covered in moist substrate of *Sphagnum* L. bogs.

Holotype. The intergeneric hybrid × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum* represents the only individual ever reported, which was not herbalized. The holotypus was

confined to printed digital images: 75 images taken by Hajnalka Kertész on the 30th June (time: 08:37:19am), 3rd of July and 30th of June 2020 and 370 images taken by Nora Anghelescu on 3rd of July and 30th of June 2020, deposited in private image data bases. Photographs were taken but no voucher material was removed from so singular a plant.

Etymology. The intergeneric name, *Pseudorhiza*, is a compound term that originates from the fusion of the first and last parts of the scientific names of two distinct genera, *Pseudo* (rchis) and (Dactylo)*rhiza*. It refers to the rare cross between the species *Dactylorhiza fuchsii* subsp. *sooana* (Borsos) Borsos and *Pseudorchis albida* subsp. *tricuspis* (Beck) E.Klein, which were the only species found in the hybrid's close proximity.

The nothospecies epithet, *nieschalkii*, was given in honor of the German botanist Albert Nieschalk (1904–1985), who discovered the hybrid × *Pseudorhiza nieschalkii* (*Dactylorhiza fuchsii* × *Pseudorchis albida*), in in 1947 in Germany at the 'New Hagen' near Niedersfeld in North Rhine-Westphalia (Eccarius, 2016), hence its potential vernacular name, Nieschalk's *Pseudorhiza*.

The nothosubspecies epithet, *sicolorum*, comes from the Latin name *Terra Siculorum* meaning *Siculorum County*, ad litteram meaning of *Siculorum*, a reference to the area where this unique orchid hybrid is endemic, which gave its vernacular name *Pseudorhiza Siculorum*. The nothosubspecies epithet, *sicolorum*, was subsequently given to this new hybrid reflecting the identity of the region, Siculorum County, that yielded the holotype and only known plant. Terra Siculorum known as the Székely Land or Ținutul Secuiesc is a historic and ethnographic area in Romania, inhabited mainly by Székelys. It includes the whole territories of Mureș, Harghita and Covasna counties and its main cultural centre is the city of Târgu Mureș, the largest settlement in the region.

Discussion

The intergeneric hybrid plant was found in the western area of Harghita-Mădăraș heath, by Hajnalka Kertész, on 30th of June 2020, at 08:30:19 (Fig 8). Its likely parentage was immediately recognised, its putative parents growing within 2 and 10 meters respectively, but its greater significance as a hybrid combination new to science was not fully elucidated at that moment. Therefore, digital images were taken by Hajnalka Kertész, but neither detailed measurements nor samples suitable for chromosomal analysis were obtained. Very soon after, digital images were sent to Nora Anghelescu who replied almost immediately, not only confirming that it was indeed a hybrid plant but also pointing out that this hybrid combination was new to science. Following an urgent request by Nora Anghelescu, several trips followed, trips during which the hybrid and the putative parents were analysed, measured and photographed in great detail.

Sadly, an attempt made by Nora Anghelescu and Hajnalka Kertész five weeks later for more careful flower and pollination studies (in July 2020) were impeded by the complete destruction of the entire 'protected' area by uncontrolled cattle grazing. The hybrid was still relocated since natural landmarks and digital photos were taken in order to precisely mark the exact placement of the hybrid within the heathland. In autumn, millimetric explants (adventitious root tips) for chromosomal counts were collected in order to fully and precisely elucidate the parental origin of the hybrid.

Helped by the supportive morphometric results, we regard the karyotypical results as reliably demonstrating that the putative hybrid is indeed the F₁ natural cross between *D. fuchsii* subsp. *sooana* and *P. albida* subsp. *tricuspis*. The status of this intergeneric hybrid as new to science was a further motivation to apply additional chromosomal analyses (as opposed to visual guesswork) in the hope of achieving a final and conclusive identification.

Contrasting pollination syndromes discussion

All species belonging to *Dactylorhiza* genus are nectar-deceit orchids [29]. Their spurs are completely devoid of nectar and consequently, these *non-rewarding* species with *nectarless flowers* do not offer any recompense to the pollinating insects. Hence, their pollination is exclusively based on *deceit* and *mimicry*. All food-deceptive orchids exploit the pre-existing plant-pollinator relationships, especially the food foraging behaviour and achieve their pollination by deception. They are known as food-deceptive orchids and this particular pollination mechanism is classified as generalized food deception mechanism [30]. Despite the fact that they are rewardless, all *Dactylorhiza* species are *allogamous* (they do not self- or auto-pollinate) and depend entirely on insects for their *cross-pollination* and *seed production*. They are usually pollinated by generalist pollinators such as bees, bumble-bees, beetles, butterflies, flies, attracted by the neighbouring nectariferous, rewarding plant species, also termed as pollinators' 'magnet-species' [31, 32]. According to the "magnet-species theory" nectarless, rewardless plants take advantage from growing in the vicinity of nectar-producing species by sharing and exploiting their pollinator community [33].

Additionally, in order to attract insects and successfully accomplish their pollination, they often mimic their nectariferous neighbours in one or more floral traits such as, inflorescence shape, flower colour, floral scents (that mimic the presence of nectar), nectar guides, spurs and pollen-like papillae. Little (1983) termed this type as 'mimicry based on naïveté' [34].

Although most authors have argued that *Dactylorhiza* in general, offer no reward to the visitor, Dafni & Woodell (1986) demonstrated that some insects refuse to leave the nectarless flowers empty handed and, instead they start chewing the sugar-rich stigmatic exudates [35]. *Dactylorhiza fuchsii*, the most intensively studied species for pollinators has been recorded as receiving visits from 35 insect species [29, 35, 36]. Having taken in consideration that the heathland was populated by numerous Hymenopterans and Dipterans, it is very likely that the dominant pollinators of *D. fuchsii* subsp. *sooana* are diurnal various species of bumble-bee (*Bombus* genus), bees (*Apis* genus) and various butterflies.

Pseudorchis on the other hand, is a rewarding genus, which attracts its pollinators with its sweet, vanilla fragrance (secreted both during the day and the night) and recompenses them with large amounts of nectar. This pollination mechanism is known as generalised food foraging behaviour mechanism [37]. As a result, *P. albida* subsp. *tricuspis* is able to attract a wide variety of diurnal pollinators such as bees, butterflies and beetles, especially during the hot summer days when the alpine plains are crowded with insects, all hungry and in search for food. Notably, our observations suggest that the scent emission increases in the evening, since the plants attracted various crepuscular moths and various species of mosquitoes, compared to a significantly lower number of butterflies and hoverflies (syrphid flies) that visited the plants during the day. The fruit set and seed production are very high in case of *P. albida* subsp. *tricuspis*, often over 90% [38, 39], which raises the possibility of spontaneous autogamy [40–42], particularly in cold alpine areas where, rather often, the pollinators are scarce.

Within the hybrid's neighbourhood, the parental species occurred in very close proximity to each other, approximately at a distance measuring 2–10 metres. This implies that the pollinating insects required a minimum travel distance between the parents, in order to successfully transport pollinaria (singular: pollinarium) between individuals and generate the hybrid. Since all three taxons display a considerable synchronicity in their flowering time, and, at least partially, may share the pollinator community, frequent exchange of pollen between the parental species was/is very likely.

Thus, the resulting hybrid proofs that even highly contrasting pollination syndromes such as generalized food deception and generalised food foraging behaviour mechanisms, are insufficient to stop the gene flow between two different orchid genera.

Since the resulted hybrid had a significantly larger labellum than *P. albida* subsp. *tricuspis* parent, we speculate that prospective pollinating insects (shred from the parental pollinator communities) will perceive a flatter and proportionally wider landing stage in the hybrid than in the respective parent. Rather often, these character shifts are capable of modifying pollinator specificity, indicating a potential evolutionary future for the hybrid. Further data will explore whether the inheritance of such specific ‘character suites’ in intergeneric hybrids, impair, neutralise or enhance the functionality of these particular novel combinations of character states [43].

In the same time, further studies will be needed to elucidate whether the hybrid is completely rewardless, similar to *D. fuchsii* subsp. *sooana* parent, or whether it is, at least partially, rewarding and secretes nectar in its, eventually, sweet vanilla-scented ‘*tricuspis*-like’ flowers.

Mycorrhizal associations discussion

The successful germination of at least one F₁ seed and its development to maturity also merit special consideration, since most of the species belonging to *Dactylorhiza* and *Pseudorchis* genera (parental genera) require a period of approximately 4 years from seed germination to first appearance above ground [44–46].

Both *D. fuchsii* [47] and *P. albida* are widely viewed as mycorrhizal generalists, able to form partnerships with many fungi, thus having a much lower dependency than obligate mycoheterotrophs [36]. *P. albida* is generally associated with a wide variety of fungi members of a polyphyletic basidiomycete group collectively called Rhizoctonia, which are typically found in photosynthetic orchids of open habitats [46]. Molecular research, based on cladistic analysis of DNA sequences, places Rhizoctonia within the family Ceratobasidiaceae [48]. Most of *Dactylorhiza* species are associated with strains of *Ceratobasidium* and *Thanatephorus* (anamorph Rhizoctonia) which have been isolated from the roots of adult individuals [46].

Since the parental species share the same mycorrhizal partners/community, it is very likely that, should they be produced, the seeds of the hybrid might fall in close parental proximity and be stimulated to germinate by the same mycorrhizal fungi. It is well-known that the seeds (in this case the hybrid seeds) fall within the close vicinity of the parental plants [46] and, by making use of the mycorrhizal fungi available, they often successfully germinate. The discussion of potential production of seed was made on the assumption that since the hybrid is a diploid (allopolyploid) individual ($2n = 40$), is very likely to produce seeds by geitonogamous pollination (self-pollination).

It was very disappointing we were unable to complete the morphological study of the flowers, fruit and seeds, as the whole area was destroyed by cattle, a few weeks after the initial identification of the hybrid was done. The violent cattle intervention resulted in the grazing of the upper half of all plants in the area. We managed to relocate the hybrid and identified its remains, namely the basal leaves and the lower half of the stem, which escaped by miracle, the hungry, voracious mouths of the herbivores.

Could this unique hybrid be regarded as a new nothosubspecies?

According to strict etiquette it became common among botanists to officially describe all newly found hybrids as nothospecies or nothosubspecies, even though most are ephemeral (and, in most cases, infertile) and thus, a very small fraction of them eventually survive to develop stable nothospecies/nothopopulations (homoploid species) [36].

As mentioned, further field investigations will be needed to validate the fertility of this hybrid (even if such F₁ plants are characterised by significant reduced fertility [36]) and the

presumed potentiality of its seeds to germinate and continue to produce new individuals beyond this first generation, F₁ which, ultimately, might lead to the establishment of a new nothopopulation. In the same time, since the chromosomal numbers of the hybrid and its parental species are very close (2n = 40, 42) it would be interesting to show whether this F₁ hybrid will be able to back-cross with either of its parental species and generate further new phenotypically distinct offspring.

Conclusions

In order to elucidate the origin of the newly discovered hybrid, × *Pseudorhiza nieschalkii* nothosubsp. *sicolorum*, we applied two different scientific methods, morphometry and chromosomal counts plus one less scientific method, botanist intuition. As data accumulated, we concluded with confidence that the plant is a natural F₁ hybrid generation that has *D. fuchsii* subsp. *sooana* and *P. albida* subsp. *tricuspis* as parents.

Nevertheless, we wonder how many genuine hybrids go unnoticed during field research and how many such cryptic hybrids reports are actually genuine hybrids.

In conclusion, we consider this novel combination an extremely fortunate find considering the exceptional geographic restriction/isolation of the parental species, in the fact that *D. fuchsii* subsp. *sooana*, exclusively confined to Harghita-Mădăraş protected area, favours mildly acid soils (moist heathland), whereas *P. albida* subsp. *tricuspis* is typically an alpine plant of soils that are moist to wet and neutral to alkaline (marshes, limestone grassland). Altogether, these contrasting requirements and the extremely reduced distribution of one parental species (*D. fuchsii* subsp. *sooana*), offer very slim chances of the parental taxons to develop sympatric populations. Harghita-Mădăraş natural reserve thus represents a unique exception.

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