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MORPHOMETRIC AND MORPHOLOGICAL ANALYSES OF *ANACAMPTIS* × *TIMBALI* NOTHOSUBSPECIES *REINHARDII* A NEW ORCHID HYBRID POPULATION TO ROMANIA

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Abstract

This is the first confirmed occurrence in Romania of a significant (notho)population of seven hybrids, Anacamptis × timbali nothosubspecies reinhardii (Ugr. ex E.G. Camus) H. Kretzschmar, Eccarius & H. Dietr., 2007, hybrids between two highly divergent species, Anacamptis coriophora (from Anacamptis coriophora group) and Anacamptis palustris subsp. elegans (from Anacamptis palustris group). The seven hybrids, very likely F1 generation plants representing a single interspecific/intrageneric pollination event, were first studied at Grădiştea Muncelului-Cioclovina Natural Park, Hunedoara County, Romania. The hybrids were phenotypically intermediate between their parental species in most of the 25 morphometric and 41 morphological characters scored, but significantly, they closely resembled Anacamptis palustris subsp. elegans parent. Additionally, pollination studies were performed. Since the parental species occurred in near proximity (at less than 1 meter 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 A. coriophora and the hybrid, which display a considerable synchronicity in their flowering time, were proved to overlap in pollinator community, very successfully sharing the solitary bees belonging to genus Lasioglossum. The presence of fruits in almost all the hybrids is another proof that they were successfully cross-pollinated. It is clear that even contrasting pollination syndromes such as generalized food deception (in the nectarless Anacamptis palustris subsp. elegans) and generalised food foraging behaviour (in the nectar-producing Anacamptis coriophora) mechanisms are insufficient to fully stop the gene flow between the two species. We set this hybrid population discovery in the context of the recent, expanding evidence of the occurrence of wild species of orchids in Grădiştea Muncelului-Cioclovina Natural Park.

***Key words**: anacamptis, hybrid, orchid, timbali, speciation, Orchidaceae.*

INTRODUCTION

This intrageneric/interspecific hybrid is a combination between two highly divergent and taxonomically controversial species *Anacamptis coriophora* (L.) R.M. Bateman, Pridgeon & M.W. Chase, 1997 (from Section *Coriophorae* (Parl.) H. Kretzschmar, Eccarius & H. Dietr., 2007) and *Anacamptis palustris* (L.) R.M. Bateman, Pridgeon & M.W. Chase subsp. *elegans* (Heuff.) R.M. Bateman, Pridgeon & M.W. Chase, 1997 (from Section *Laxiflorae* (Soó & Keller) H. Kretzschmar, Eccarius & H. Dietr., 2007). They are now members of the newly supplemented genus *Anacamptis* Rich., 1817 (previously, these two parental species were members of the genus *Orchis* Tourn. ex L. 1753; following extensive molecular analyses, recently, they have been moved into *Anacamptis* genus).

The generic name, *Anacamptis*, originates in the Ancient Greek word anákamptein, which ad litteram means bent-backward, a reference to the bent-backward (reflexed) tips of the pollinia (the sacs of pollen), a characteristic of this genus. The hybrid (nothospecies) epithet, *timbali*, was given in honour of Édouard-Pierre-Marguerite Timbal-Lagrave (1819-1888), a French pharmacist and botanist who specialized in the flora of southwestern France, including the Pyrénées and Corbières mountains, hence its vernacular name, Timbal's Anacamptis.

The hybrid (nothosubspecies) epithet, *reinhardii*, was given in honour of Reinhard Gustav Paul Knuth (1874-1957), a German taxonomist and botanist hence its other possible vernacular name, Reinhard's Anacamptis Hybrid. Taxa with the specific or nothosubspecific epithet, *reinhardii*, commemorate his name. The accepted scientific name was established in 2007, as *Anacamptis* × *timbali* nothosubspecies *reinhardii* (Ugr. ex E.G. Camus) H. Kretzschmar, Eccarius & H. Dietr., Orchid Gen. Anacamptis, Orchis & Neotinea, ed. 2: 428 (2007). Its basionim (the first name ever given to a taxon) is *Orchis* × *reinhardii* Ugr. ex E.G. Camus, Monogr. Orchid.: 230 (1908). This nothosubspecies belongs to nothospecies *Anacamptis* × *timbali* (Velen.) H. Kretzschmar, Eccarius & H. Dietr., 2007, a member of genus *Anacamptis* Rich., 1817, subtribe Orchidinae Dressler & Dodson, 1960/Verm., 1955, tribe Orchideae Dressler & Dodson, 1960/Verm. 1977, subfamily Orchidoideae Lindl., 1826, family Orchidaceae Juss., 1789. Since we found at least *6 different individuals*, the hybrids have been given the *nothospecies status*. This is the first nothopopulation of *Anacamptis* × *timbali* nothosubspecies *reinhardii* ever mentioned in Romania. Consequently, we strongly propose this hybrid as a new addition to/candidate for the Romanian flora. Moreover, we believe is imperious to put the entire area under strict protection.

MATERIALS AND METHODS

Location description

The extreme rarity of this crossing is due to the fact that both parents are rather rare and to find them together is rather exceptional. The preferred habitat is a seasonal, full sun, grassy, alkaline marsh, periodically prone to flooding. The soil composition may be a mixture of calcareous alluvium that provides a substrate for the entire marsh, sand, clay, etc., derived from the adjacent slightly well-drained areas and dunes. The surface of the soil was moist but not water logged (marshy meadow). The sample sites for all three taxa were located within a few metres of the ecotone between the marsh and back landward. To our surprise, the area was already being developed for housing, whereas the adjacent marsh has retained much of its original flora. The vegetation of this wetland reserve is dominated by herbaceous swampy species. No other orchid species were noticed to occur in the area. The researched area covered approximately 1 square kilometre. It was reported that it is periodically grazed by cattle and three weeks after this study was performed, it was almost completely mowed. *Anacamptis palustris* subsp. *elegans* occurred in significant numbers in the marshy parts of the area, reaching approximately 150-200 individuals. The foetid-smelling *Anacamptis coriophora* grew immediately adjacent to the hybrids, in the ecotone and the drier parts of the swamp. Its numbers were significantly higher, probably encountering several thousand plants (2,000-3,000). All 6 hybrids were growing in the close vicinity of *Anacamptis coriophora*, within the drier parts of the swamp. The distances between the hybrids and *Anacamptis coriophora* parent were very short, in some cases measuring only 5-10 centimetres, in other cases slightly higher, just over 1 meter. The distances between the hybrids and *Anacamptis palustris* subsp. *elegans* were significantly longer, from 1-2 metres up to 20-30 metres or more, if we take in consideration the distance to the edges of the swamp, where scarce *Anacamptis palustris* subsp. *elegans* groups were found.

Flowering time

The flowering times of all three species (parents and hybrid) overlapped almost entirely. *Anacamptis coriophora* parent was the first to flower. Approximately one week later, *Anacamptis palustris* subsp. *elegans* parent came into bloom. The hybrids flowering time seemed to be intermediate between parents, although, at the time the studies were performed, all three species were at the peak of anthesis. In some parts of the swamp, some *Anacamptis coriophora* individuals were slightly off the peak of anthesis.

Parental species description

Anacamptis coriophora (L.) R.M. Bateman, Pridgeon & M.W. Chase, 1997

The specific epithet, *coriophora*, originates in the Ancient Greek words khórion (crust, insect) and phór(os) (to carry, to transport,), ad litteram meaning smelling like a bug, a reference to the unpleasant, strong scent that its flowers usually emit, which resemble that of some species of bugs, hence its vernacular name, the Bug Orchid. The flowers can display considerable variation, particularly in the shape and colour of the labellum and the helmet. The commonest variant has a dark-reddish or brownish-purple helmet and a pale-coloured labellum, but the helmet can also be green flecked with dark brown, greenish-white, or reddish purple. The colour of the labellum can vary from whitish with extremely faint spots to intense purple-brown, with strong dark-purple spots. The leaves are linear, linear-lanceolate or lanceolate, numerous, standing straight up, most at the base. Inflorescence are prolonged-cylindrical. The bracts are linear-lanceolate (Kuhn et al., 2019). The flowers are small, coloured in greenish-red to brown-purple hues. The petals are pointed, gathered in a solid beak-shaped helmet. The labellum is bent down and backwards, trilobed, olive green colour, at the base whitish with purple spots. The spur is tapering, bend downwards, shorter than the ovary. The flowers are rewarding, abundant in nectar and usually many are pollinated (Claessens & Kleynen, 2011). Diploid chromosome number: $2n = 36, 3$

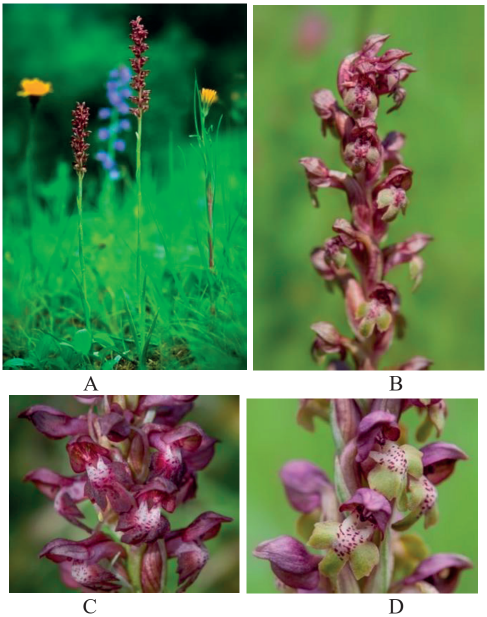

Figure 1. *Anacamptis coriophora.* Entire plant (A) and details of several inflorescences with different chromatic variations (B-D) Photos A-D © N. Anghelescu originals

Anacamptis palustris **(L.) R.M.Bateman, Pridgeon & M.W.Chase subsp.** ***elegans*** **(Heuff.) R.M.Bateman, Pridgeon & M.W.Chase, 1997**

The infraspecific epithet (subspecies epithet), *elegans*, originates in the Old Latin word ēlegāns (tasteful, select), ad litteram meaning delicate, refined, a reference to the elegant, tall inflorescences of this subspecies, which bears beautiful, large flowers, hence its (potential) vernacular names, the Elegant Anacamptis, the Elegant Swamp Orchid or the Elegant Marsh Orchid. It is a tall, beautiful and imposing plant that may reach 50-100 centimetres in height. The lanceolate leaves are larger and longer, the flower bracts are longer than the ovary/receptacle (Delforge, 2006). Similar to *Anacamptis coriophora, Anacamptis palustris* subsp. *elegans* is a wintergreen species. They emerge in autumn and wither after anthesis. The flowers are conspicuously and more or less uniformly purple and show little variation. Most of the plants can reach 60-90 centimetres, but oscillates most often between 10 and 25 centimetres. Its lanceolate erect leaves are located at the base of the plant and others smaller, not very visible, are fixed on the stem (stem-leaves). The dense inflorescence forms a pyramidal spike of tight flowers. The deep pink-purple flowers, whose colour can vary from light pink to purple, are very rarely white (Presser, 2002). The labellum, clearly trilobed, provided with two protruding ridges at the base, forms towards the back, a filiform spur. The plant does not have nectar, the attraction of the butterflies for the latter is therefore a decoy. As it is a question of ensuring the fertilization, the morphology of the flowers is well adapted to the proboscides of the Lepidoptera, which may be diurnal or nocturnal (Pridgeon et al., 2001).

Diploid chromosome number: $2n = 36, 42$

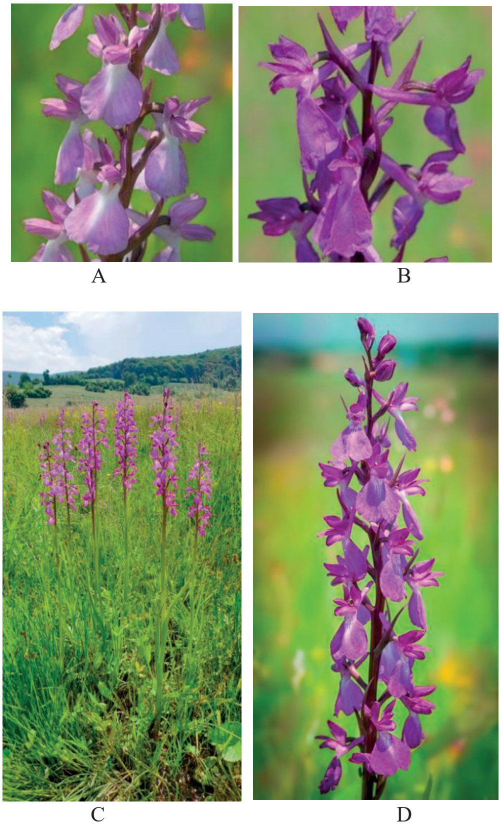

Figure 2. *Anacamptis* palustris subsp. *elegans.* Details of hypo- and hyperchromatic chromatic inflorescences (A-B), entire plant (C) and detail of a full inflorescence (D). Photos A-D © N. Anghelescu originals

General descriptions of the hybrids

The six hybrids closely resembled each other morphologically in size and shape, suggesting that they might have the same parental origin. Primary hybrids (F_1 generation) are mainly much scarcer than their parents and, in general, appear phenotypically intermediate between the parental species.

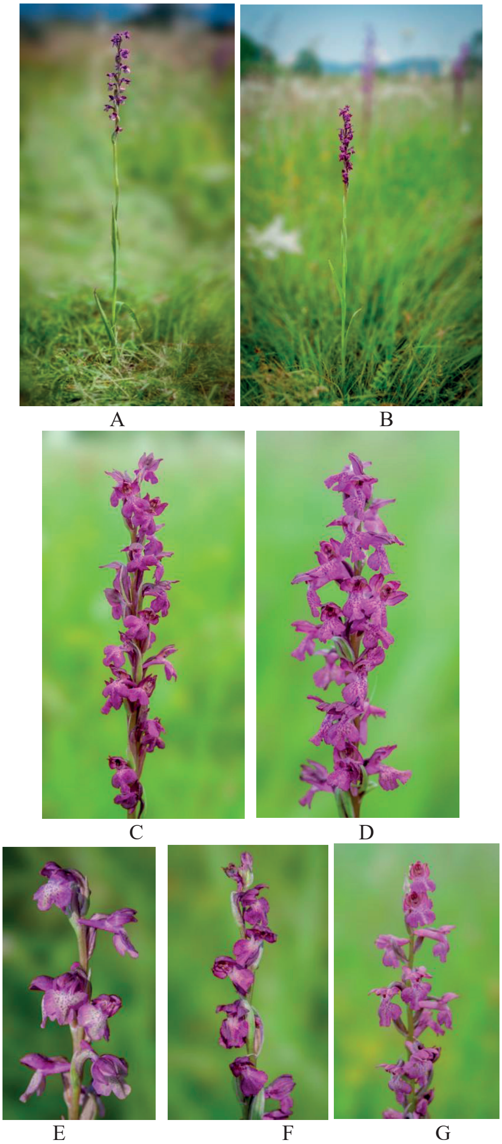

Figure 3. *Anacamptis* × *timbali* nothosubspecies *reinhardii*. Entire plants (A-B) and details of several inflorescences that present different polymorphic (shape, size, colour) variations (C-G). The hybrids are very tall, a feature inherited from their *Anacamptis palustris* subsp. *elegans* parent. The inflorescences are laxer with smaller flowers, resembling more those of *Anacamptis coriophora* parent. Photos A-G © N. Anghelescu originals

Figure 4. Morphological comparisons of *Anacamptis* × *timbali* nothosubspecies *reinhardii* and parents (A-D). The illustrations show the intermediate characters of the hybrids, compared to those of its parents. Photos A-D © N. Anghelescu originals

RESULTS AND DISCUSSIONS

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 most cases, the phenotypical characters/traits of the hybrid appear intermediate between the parental species in the majority of morphological characters.

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 (Bateman & Hollingsworth, 2004).

In order to describe the plants as comprehensive as possible, a wide range of characters are taken in consideration and biometrically/morphometrically analysed in millimetres, unless otherwise stated (Jacquemyn *et al*., 2012).

The quantitative measurements encompass all organs except the tubers and gynostemium.

Measurements are examples of several parental plants and of hybrid no. 4 & 5 (in all individuals measured, it has to be mentioned that older flowers found at the bases of inflorescences are better developed than the younger flowers found in the upper half).

Table 1. Morphometric comparison of the parental species and potential hybrids. The quantitative measurements (in mm unless otherwise stated) encompass all organs except the tubers and gynostemium

Vegetative & Floral Organs	Characters / Features (milimetres)	*Anacamptis palustris* subsp. *elegans*	*Anacamptis × timbali* nothosubspecies *reinhardii*	*Anacamptis coriophora*
1. Stem & Inflorescence				
	Overall height	560/600	550	200
	Stem diameter	6.3	5.5	3.4
	Stem anthocyanins	moderate/strong	moderate/strong	low/absent
	Inflorescence length	180/250	180	70/90
	Number of flowers	24/30	21	48/53
2. Leaves				
	Distribution of sheathing leaves on stem	even	basal/even	basal
	Longest leaf posture	erect	slightly recurved/erect	slightly recurved/erect
	No. of sheathing leaves	3	3	4/8
	No. of leaves non-sheathing	3	3	2/5
	Length of longest leaf	200/250	150/180	22/72
	Width of longest leaf	30/38	20/30	18/20
	Outline shape of longest leaf	linear-lanceolate	linear-lanceolate	linear-lanceolate
	Leaf conduplicate	strong	moderate	moderate
	Apex hooding	strong	moderate	moderate
	Leaf colour	vivid/light green	deep green	deep green
	Leaf dorsal side	light green	light green	darker green
	Leaf ventral side	green	green	greyish green, veined
	Leaf margins	entire	entire	entire
	Leaf markings	unmarked	unmarked	unmarked
	Upper leaves	bract-like	cauline	bract-like
3. Bracts & Ovary				
	Length of basal bracts	33/36	39/45	9/12
	Width of basal bracts	5/8	7/8	3/3.2
	Length of floral bracts	20/22	22/23	2.2/2.3
	Width of floral bracts	3.2/3.6	3.8/4.2	2.1
	Texture of bracts	robust	robust	membranous
	Bract anthocyanins	strong	absent/moderate	Moderate/strong
	Marginal wall thickness	thick	thick	thin
	Ovary length	16/20	12/15	5/8
	Ovary diameter	2.3/4	2.5/3	2.3
	Ovary anthocyanins	absent/moderate	moderate	strong
4. Sepals & Lateral Petals				
	Lateral sepals position	near-erect	near-erect	erect
	Lateral sepals connivent	no	yes	yes
	Sepal fusion from base (%)	40	60	70/80
	Sepal apex	Oval/blunt	acute	acute
	Sepal iridescent green pigment	absent	present	present
	Median sepal length	11.1/12.8	9.2/9.9	8.1/8.7
	Median sepal width	3.2/5	2.9/3.2	1.3/1.6
	Lateral sepals width	3.2/4.6	2.3/2.9	1.4/1.6
	Lateral sepals length	12.2/12.6	7.9/8.1	8.3
	Lateral petals width	3.3/4.3	2.8/3	1.4/2
	Lateral petals length	7.9/11.2	7.3/7.8	5.3/6.2
5. Labellum				
	Outline shape	elongated-oval, nearly flat	circular/heart shaped, nearly flat	longitudinal-oval, lobesfolded backwards
	Lobes	nearly entire	trilobed	deeply trilobed
	Median lobe length	12.4/13.4	7.8/8.9	7.1/8.9
	Lateral lobes length	11.8/12.1	7/7.8	5.6/6

Vegetative & Floral Organs	Characters / Features (milimetres)	*Anacamptis palustris* subsp. *elegans*	*Anacamptis × timbali* nothosubspecies *reinhardii*	*Anacamptis coriophora*
	Sinuses separating the three lobes	shallow	medium/deep	very deep
	Width	16.5/17.8	9.3/11	7.3/8
	Lateral lobe reflexion	absent	absent/moderate	strong
	Central lobe apex	shallow, bilobed, flat	prominent, entire, flat	prominent, strongly recurved
	Central lobe width	4.2/5.3	4.2/4.6	1.6/2.1
	Base colour of labellum	whitish, purple-dotted	pink to light purple, purple-dotted	whitish, grooved, red-dotted
	Centre colour	whitish, elongated streak, purple-dotted	whitish -yellowish-pinkish, convex, slightly to strongly uniformly dotted	whitish, grooved, convex, dark-red dotted
	Margins colour	deep-purple, uniform	purple, brownish to very dark-purple	dark-red, green, brown
	Markings type	purple stripes	dense purple spots	dark red or brown spots
	Surface markings papillate	no	moderately	strong
	Markings distribution in centre	linear-vertical	circular	circular
	Markings contrast	weak	strong (weak at times)	strong
	Lateral lobe indentation	entire, scalloped	entire/moderate	prominent
6. Spur				
	Spur length	15.2/17	8.2/8.9	7.5/8
	Spur shape	cylindrical thin, ascendant	cylindrical, thick, horizontal	conical, thick, slightly descendant
	Spur width entrance	3.2/3.6	4.6/4.9	4.8/5/4
	Spur width halfway	1.2/1.5	2.6/2.9	2.1/3.2
	Spur down-curvature	none	none/slightly down-curved	down-curved
7. Nectar				
	Presence	no	?	yes
	Amount	-	?	abundant
8. Smell				
	Presence	vaguely fruity	vaguely bug-like	Strong, bug-like, carrion

Morphometric comparisons

Habitus: The hybrids are very tall and sturdy, reaching up to 60 centimetres, a feature inherited from *Anacamptis palustris* subsp. *elegans*.

Stem: Is moderately to strongly purple washed. The presence of anthocyanins is also a feature inherited from *Anacamptis palustris* subsp. *elegans*.

Leaves: Two out of the 6 hybrids show larger cauline leaves, erect, lanceolate, sheathing the stem, a feature inherited from *Anacamptis palustris* subsp. *elegans*. The other 4 hybrids resemble more *Anacamptis coriophora*, by presenting 2-3 basally concentrated leaves and 1-2 cauline leaves, a lot smaller, sheathing the stem. Also, the colour of the leaves is intermediate between the parents, the hybrid presenting slightly darker leaves than *Anacamptis palustris* subsp. *elegans* which has vividly to light green leaves.

Flower: The flower sizes of the hybrids lie between that of both parents. They show a variety of intermediate sizes (they differ very slightly) and range mostly in the median range of the parental sizes (length x width).

Bracts: The bracts are very variable in size. They are shorter to almost equal to the ovaries like in *Anacamptis coriophora*. In hybrid no. 3, the bracts are very long, almost twice as long as in other hybrids, and longer than in both parents. This is a feature which shows a hybrid enhanced character.

Lateral Sepals & Petals: The sizes are again, intermediary between the parental species.

Helmet/hood: the lateral petals and sepals that construct the helmet are not linked and the helmet opens slightly at the crown, in intermediary condition between the parental species.

Labellum: The morphology of the labellum is particularly interesting as the parents differ considerably in the labellum size and shape. The labella of the hybrids are all different in shapes, lobe depth and colour. One of the hybrids (hybrid no. 4) resembles more *Anacamptis palustris* subsp. *elegans* (has very shallow lobe separation and the lobes are equal, almost undefined), presenting a shallower labellum indentation. Hybrid no. 6 lies at the other extreme: it presents a deeply trilobed labellum resembling more *Anacamptis coriophora* (has a narrow, prominent central lobe and allows all three lobes to reflex). The rest of the hybrids are intermediary between these two. In all 6 hybrids, the lobes are not reflexed and the labellum appears almost flat.

Consequently, we speculate that a prospective pollinating insect will perceive a flatter and proportionally wider landing stage in the hybrid than in the parent. Moreover, the purple labellum colour of the hybrid, represents a combination of anthocyanin pigments, which is intermediate between the dark-red of *Anacamptis coriophora* and the vivid purple of *Anacamptis palustris* subsp. *elegans*.

A particularly interesting phenomenon of overexpression of pigmentation was observed in 4 hybrids (especially in hybrid no. 4). In all cases, the flowers become dark, deep-purple, different from either parent. The high density of floral anthocyanins is evident only in the hybrids, relative to either of the parents. This interesting example of a hybrid enhanced character reinforces the supposition that the over-expression of pigmentation, which will result in lower reflectivity of the flowers is more commonly observed in hybrids between distantly related species.

The increased hybrid vigour in some particular features, which became superior to both parents, is called the 'heterosis effect' (Kretzschmar *et al.,* 2007). These character shifts are capable of modifying pollinator specificity, indicating a potential evolutionary future for the hybrid. These data will show and explore whether specific patterns of inheritance of specific characters suites and determine whether particular novel combinations of character states (or novel states) in such hybrids, impair, neutralise or enhance with respect to functionality. On the other extreme, lies hybrid no. 2, which has a lot less anthocyanin on the labellum, shifting more towards *Anacamptis coriophora* in this respect.

Labellar markings: In all hybrids, the markings of the labella present a heart-shaped pattern of distribution (more circular), strikingly resembling the distribution in *Anacamptis coriophora*. None of the hybrids inherit the longitudinal whitish streak of *Anacamptis palustris* subsp. *elegans*. Also, the texture of the central area of the labellum is strongly papillose, reminding of the brownish-red papillose spots of *Anacamptis coriophora* feature.

Spur: The spur is generally long (but shorter than the ovary), cylindrical, thick and horizontal, in most of the cases. In one case, hybrid no. 5 is conical, with a pointed tip, resembling *Anacamptis palustris* subsp. *elegans*. The others are rather thick showing a strong *Anacamptis coriophora* influence. Only one of the hybrids, hybrid no. 1 has a slightly downward pointing spur.

Hybridisation within *Anacamptis* genus

According to Goulet & Hopkins (2017), the term *hybridisation* is rather controversial and needs a new, updated definition. Often, hybridisation is only considered between species, but '*from a genetic point of view, interspecific hybridisation is only a special case of a much more widespread phenomenon*' (Stebbins, 1950). Therefore, especially when referring to orchidology, it would be useful to redefine the phenomenon of hybridisation independent of species strict definitions, which stated that '*the species is the basic category of biological classification, composed of related individuals that resemble one another and who are able to breed only among themselves'*.

In this article, we will adopt its broader definition given by Harrison (1990), in which '*hybridisation is a cross between individuals from separate populations that differ in one or more heritable traits'*. Defining hybridisation independent of the species distinction, elegantly circumvents the problem of species definition. 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.

In 1786, in his treaty Disquisitio de sexu plantarum (A Dissertation on the Sexes of Plants), Carl Linnaeus (1707-1778) first suggested that new species arose [mainly] by hybridization, thus rejecting the notion of species 'immutability' or the incapacity of species to change through time (Coyne & Orr, 2004). A new hybrid lineage is formed through parental genome mixing. 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 (Mayr, 1942). As a result, hybridisation was shown to have a significant role in speciation, generating new species with better genetic, adaptive variation (Arduino et al., 1996).

By definition, hybridisation is the crossing of two different genotypic parents, parent generations $P_1 \times P_2$. The genes from P_1 & P_2 exist in the first subsequent generation, named F_1 (Soltis & Soltis, 2009). They will be present in the hybrid genotype and can be dominant, recessive or intermediate (Ramsey & Schemske, 1998). 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.

Scarcity of orchid hybrids: In the wild, the maintenance of species integrity has major importance. In the case of sexually compatible sympatric populations, species integrity depends upon several reproductive barriers that secure the reproductive isolation between species. They are classified in:

- pre-mating barriers - spatial segregation, phenology (scientific study of cyclical biological events, such as flowering periods, breeding, seed production, in relation to climatic conditions) and pollinators;
- post-mating barriers - significant differences in their haploid chromosome number (n), fruit abortion, seed unviability, hybrid unviability and hybrid sterility (Bateman & Hollingsworth, 2004).

Because of the sequential action of these isolating mechanisms, it is generally assumed that pre-mating isolation barriers are more important to reproductive isolation than post-mating barriers, although conclusive evidence for this is still largely lacking (Scopece et al., 2008).

Within *Anacamptis* genus, hybridisation among various sympatric species was shown to be quite successful, as the pre-mating barriers barriers against hybridisation are otherwise low. Between some species, hybrid swarms often appear, e.g. between *Anacamptis morio* x *Anacamptis papilionacea*.

Space segregation: In the case of *Anacamptis palustris* subsp. *elegans* and *Anacamptis coriophora,* the space segregation was extremely low, very often measuring less than 5 centimetres. Also, on this particular location, the two sympatric orchid species occurred in high density, in very large numbers - dozens of dozens of *Anacamptis palustris* subsp. *elegans* were surrounded by a high density of hundreds (or even thousands) of *Anacamptis coriophora*.

Phenology: They also display considerable overlap in flowering time: whereas flowering in *Anacamptis coriophora* starts at the beginning of May and lasts until the beginning of June (the latest), *Anacamptis palustris* subsp. *elegans* usually starts flowering two weeks later (mid-May) and flowering lasts until mid-June. On the date the hybrids were found, on 5th-9th of June, both parents were in full flower, although *Anacamptis coriophora* was slightly over the peak of anthesis, reaching the late stage of flowering. The majority of individuals had the lower flowers of the inflorescences already withered, with thick ovaries developing into fruits. *Anacamptis palustris* subsp. *elegans* and *Anacamptis × timbali* nothosubspecies *reinhardii* were both approaching the peak of anthesis. Is needs to be determined whether the hybrid actually flowers 1-7 days before or after its parents.

Chromosome number: These findings also comply with the fact that the parental species have the same chromosome number, $2n = 36$, allowing them to cross relatively easily.

Pollination: All species of *Anacamptis* genus are nectar-deceit orchids. The spurs of those species do not produce any nectar, and as a consequence, they do not reward their pollinators with any food bodies /substances.

They are known as food-deceptive orchids and this particular pollination mechanism is classified as generalized food deception mechanism (Jersáková et al., 2006).

In order to attract insects and successfully accomplish their pollination, they usually grow among rewarding plant species, which they very often mimic in one or more floral traits such as, inflorescence shape, flower colour, floral scents (that mimic the presence of food - nectar), nectar guides, spurs and pollen-like papillae. Little (1983) termed this type as 'mimicry based on naïveté'.

But there is one exception within this non-rewarding genus: the foetid *Anacamptis coriophora*, which is a rewarding species that produces abundant amounts of nectar in its spur and recompenses its pollinators with this very nutritious food. This pollination mechanism is known as generalised food foraging behaviour mechanism (Galizia et al*.*, 2005). It also attracts a large variety of visitors with its poignant and heavy smell that usually reminds of carrion (dead flesh) or bug-odour (hence its vernacular name, the Bug Orchid).

All food-deceptive orchids exploit the preexisting plant-pollinator relationships, especially the food foraging behaviour and achieve their pollination by deception. They are also generalist pollinators, usually pollinated by bees, bumble-bees, beetles, butterflies, flies, and share most of their pollinators with all the neighbouring nectariferous, rewarding plant species (Claessens & Kleynen, 2011).

As mentioned, the parental species occurred in the very near proximity of each other, sometimes at a distance measuring less than a few centimetres. This implies that the pollinating insects required a minimum travel distance between the parents, in order to generate the hybrids. Since *Anacamptis coriophora* and *Anacamptis palustris* subsp. *elegans* may, at least partially, overlap in their pollinator community and display a considerable synchronicity in their flowering time, frequent exchange of pollen between the parental species was very likely.

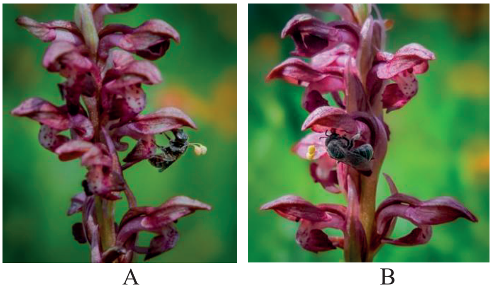

Figure 5. *Anacamptis coriophora* pollinated by solitary bees belonging to genus *Lasioglossum* Curtis, 1833, Family Halictidae Thomson, 1869 (A-B). Photos A-B © N. Anghelescu originals. Insect ID: Prof. Bogdan Tomozei

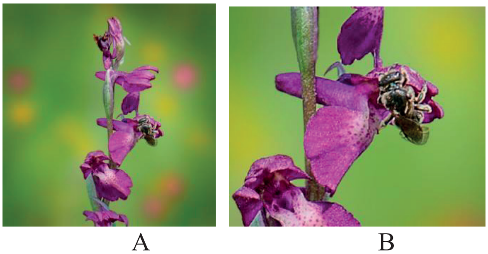

Figure 6. *Anacamptis* × *timbali* nothosubspecies *reinhardii* pollinated by the solitary bees belonging to genus *Lasioglossum* Curtis, 1833, Family Halictidae Thomson, 1869 (A-B). Photos A-B © N. Anghelescu originals. Insect ID: Prof. Bogdan Tomozei

This is one important example of strong overlap in pollinator community between *Anacamptis coriophora* & *Anacamptis* × *timbali* nothosubspecies *reinhardii.* It shows that the hybrid may, very successfully share at least on very efficient pollinator, the solitary bees belonging to genus *Lasioglossum* (see image), with one of its parents. The presence of fruits in almost all the hybrids is another proof that they were successfully pollinated.

Fruit & Seed: In some of the hybrids fruit was apparently already forming. Fruit set and seed formation are two of the most important post-mating reproductive barriers. The fact that the fruit was already developing may demonstrated that the hybrids are fertile (allogamous or, very scarcely, facultatively autogamous). Whether the seeds are able to mature and successfully germinate (develop beyond the protocorm stages), remains to be clarified. Seed formation will be of primary interest in our further studies. The dust-like seeds travel via air currents even further, thus conquering new territories and establishing new populations. It is well known that biology dictates that the pollinia are the viable means of achieving gene flow within populations and the seeds are the viable means responsible of establishing new populations. Nevertheless, distribution of pollen is limited to the pollen viability and endurance of the pollinating insects (Bateman & Hollingsworth, 2004).

Mycorrhizal Associations: According to our observations, all the hybrids were found to grow in the near proximity of *Anacamptis coriophora* plants, which occurred in high densities and surrounded them. Rather often, the distances between the hybrids and *Anacamptis coriophora* were only less than a few cm. On the other hand, the distances between the hybrids and *Anacamptis palustris* subsp. *elegans*, ranged from 3-5 metres up to 30-50 metres. Therefore, the hybrids were intimately admixed with one parent but were separated from the other. This may be in favour of *Anacamptis coriophora* of being the mother or the seed carrier, as it is well-known that the seeds (in this case the hybrid seeds) fall within the close vicinity of the maternal parent and, by making use of the mycorhizal fungi available, they successfully germinate.

Maternity-Paternity Testing: As many other hybrid studies show, within a hybrid population, some first-generation hybrids (F_1 hybrids or direct hybrids), may have 'inversed' same parents.

In our small hybrid population, this phenomenon of 'inverse parenting' can be translated as follows:

- some hybrids may have *Anacamptis coriophora* as mothers (*Anacamptis coriophora*♀ - ovule donors and seed carriers),
- others may have *Anacamptis coriophora* as fathers (*Anacamptis coriophora*♂ - pollen donors). Once emasculated, the *Anacamptis coriophora*♂ fathers may become *Anacamptis coriophora*♀ mothers, if pollinia of an *Anacamptis palustris* subsp. *elegans* plants successfully lands on their stigma, thus generating hybrid seeds. The same is valid for the *Anacamptis palustris* subsp. *elegans* reproductive partners. This suggests considerable mobility of the pollinia, seeds or both across the ecotone, rendering the potential results of 'maternity/paternity testing' especially intriguing.

CONCLUSIONS

Hybridisation evidence supports the recent expansion of the genus *Anacamptis*. The new hybrid described in this report represents a cross between an exceptionally small-flowered species producing nectar and one of the largest-flowered orchid species in the genus *Anacamptis*, which is completely devoid of nectar.

It is clear that even these contrasting pollination syndromes (generalized food deception & generalised food foraging behaviour mechanisms) are insufficient to fully stop the gene flow between the two species. Together, the hybrids formed within the genus *Anacamptis*, provide further evidence of the genetic cohesion of this genus.

We set this hybrid discovery in the context of the expansion of the newly occurring evidence of the occurrence of wild species of orchids in Gradistea Muncelului-Cioclovina Natural Park.

Future research will imply extensive:

- molecular analyses to confirm the identity of the plants as hybrids;
- molecular analyses to distinguish between maternal and parental species for each hybrid (*Anacamptis coriophora*♀ × *Anacamptis palustris* subsp. *elegans*♀ or *Anacamptis coriophora*♂ × *Anacamptis palustris* subsp. *elegans*♂).

The value of more careful morphological and molecular investigations will reveal the amount and direction of gene flow in orchids:

- analyses of specific enhanced characters (heterotic characters) in the hybrids relative the parental species;
- analyses of the potentiality of seed germination and determine if the hybridisation may continue beyond the first generation, F_1;
- the clarification of whether the area is either private property or may fall under the protection of the park.

Observation: the whole area was mowed probably before the seeds were able to fully mature.

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