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## Embryo structure, seed traits, and productivity of relict vine *Aristolochia contorta* (Aristolochiaceae)

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### ABSTRACT

The herbaceous vine *Aristolochia contorta* (Aristolochiaceae) is a rare plant with a fragmented area in East Asia. Confocal laser-scanning microscopy and stereomicroscopy were employed to examine the seed and embryo structure. This is the first research on embryo anatomy in *Aristolochia*. Shape and structure of this embryo may be phylogenetically significant. The seed structure of *A. contorta* is similar to that of other *Aristolochia* species. Considerable variation is found for productivity traits; the average seed set is rather low (26.7%). The studied seed and embryo traits of *A. contorta* are found to be variable, but may be potentially informative at the genus and species level. The restricted productivity and reduced population size threaten the continued survival of *A. contorta* and, perhaps, the butterfly that depends on it.

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### Introduction

The birthwort *Aristolochia contorta* Bunge, a rare plant and Tertiary relict, is an herbaceous vine inhabiting Northeastern China (Huang et al., 2003), North Korea and Japan (Kharkevich, 1987). The species reaches the northern boundary of its range in the southern part of the Russian Far East (Kharkevich, 1987). The plants grow at an uneven density within isolated small populations, and their natural renewal is very poor (Nakonechnaya et al., 2010). The rhizomes and fruits of *A. contorta* are used in Tibetan medicine; in particular, the fruits are used to treat tumors (Tiulin and Bakina, 1984). The species is recorded in the “Red Book of the Primorsky Krai” as a vulnerable species (Nesterova, 2008). The species is in desperate need of conservation and restoration of its natural populations, both for reasons as being the potential source of medicinal preparations and as an unique component of the biocenoses where it occurs. There, this plant is a key member of the ecosystem, being the only source of food for the relict butterfly *Sericinus montela* Gray (Sviridov, 1983). Knowledge about the plant's reproduction peculiarities may provide essential information on which to base conservation management. However, other than fragmented data on its fruit and seed morphology, little is known about reproductive traits and seed production capacity of *A. contorta* (González and

Stevenson, 2002; Huang et al., 2003; Nechaev and Nakonechnaya, 2009; Nesterova, 2008). Our observations in the natural habitats of *A. contorta* suggested poor seed yields as possible factors for the declining natural renewal (Nakonechnaya et al., 2010).

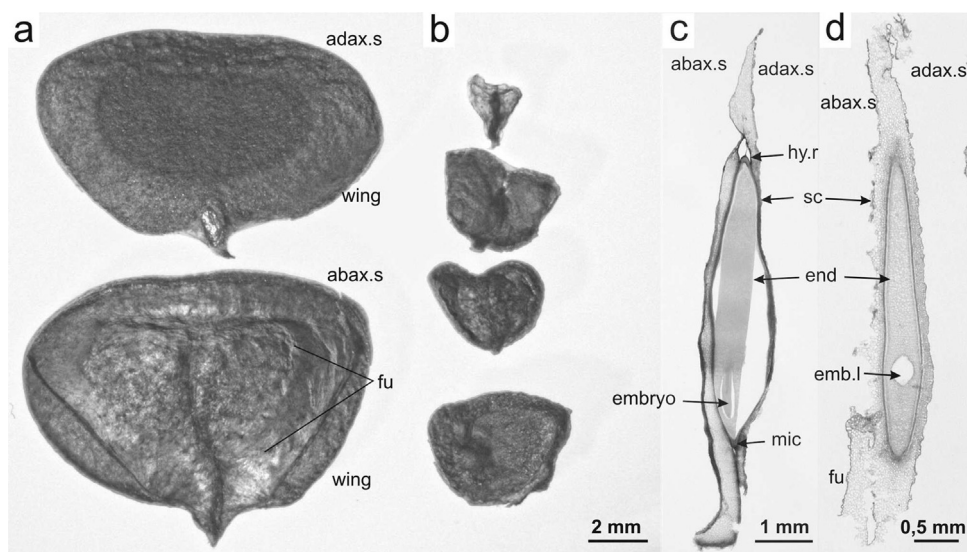
In this work, we studied the morphology and anatomy of the seed, and in particular the embryo structure, and evaluated the reproductive success of *A. contorta* by estimating the seed production per fruit and seed/ovule ratio (seed set). This is the first detailed research on the embryo anatomy in the genus *Aristolochia*, since before few only descriptions of embryo morphology for the genus have been made. According to the classification key of seed morphology by Martin (1946), embryos of *Aristolochia* have a “linear” morphology. We know of four reports in the literature to date in which the embryos of different *Aristolochia* species were characterized as underdeveloped (Adams et al., 2005a,b, 2011; Alves-da-Silva et al., 2011), and these published studies are mainly concerned with the size, development and growth of the embryo (Adams et al., 2005a,b, 2011; Alves-da-Silva et al., 2011; Johri and Bhatnagar, 1955; Martin, 1946; Mohana Rao, 1989).

### Materials and methods

Mature fruits of *Aristolochia contorta* were collected in the valleys of the Razdolnaya (20 vines) and Steklyanuha (15 vines) Rivers in the southern region of the Russian Far East in 2010. The number of seeds per fruit was determined by randomly sampling 24 mature fruits. Viable (developed, relatively large and obviously filled) seeds could be readily distinguished from aborted (obviously

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**Fig. 1.** Seed morphology of *Aristolochia contorta*: a – viable seed, adaxial view (on top), abaxial view (below); b – aborted seeds; c – longitudinal section of the seed; d – cross-section of the seed (abax.s. – abaxial side, adax.s. – adaxial side, wing, embryo, mic. – micropyle, end – endosperm, hy.r – the remnants of hypostase, emb.l. – embryonic locule, fu – adnate funiculus, sc – seed coat).

empty, 2–4 mm wide) seeds (Fig. 1b). Our germination experiments indicated that most of the seeds scored as “developed” or “filled” were, in fact, viable (Nakonechnaya, unpublished).

The following reproductive traits were determined: viable seeds per fruit; aborted seeds per fruit; and their sum (viable + aborted), which was considered to equal the total initial number of ovules. The seed set (seed/ovule ratio) was calculated. The seed size was determined for the developed seeds of 24 fruits (230 seeds). Three sets of 100 filled and aborted seeds were weighed. We determined the embryo size of a total of 50 seeds after removing the seed coat. All of the external measurements were obtained using a stereomicroscope Stemi 2000C (Carl Zeiss) and software AxioVision 4.8.

The study of the seed and embryo structures was performed using the modified clearing technique with a mixture of methyl salicylate (Sigma) and dibutyl phthalate (Sigma) (Grame, 2001). The autofluorescence of the samples was detected using a LSM 510 META confocal laser-scanning microscope (Carl Zeiss). Slices of a thickness of 10–40 μm were prepared. The images of the embryos were obtained after excitation at 488 nm and emission at 522 nm using a 505 nm LP filter under 40× and 100× objectives. The intensity of the argon laser was 4% of the maximum value. The single images and 3-D series (Z-stacks) were analyzed using LSM 510

Release 4.2 и ZEN 2009 LightEdition software by summing single optical slices (maximum projection). We stained the slices with safranin, hematoxylin and alcian blue (Barykina et al., 2004).

## Results

### Seed size

Viable seeds differed in their shapes and sizes (Table 1). The width of the *A. contorta* seeds exceeds the length. The mass of the viable seeds was the most stable trait and exhibited much less variation (CV = 3.6%) than the mass of the aborted seeds (CV = 27.1%). The coefficients of variation for the metric traits (length and width) were similar (from 12 to 18%) for the seeds and embryos.

### Seed structure

The seeds of *A. contorta* are dark brown, dry, flat, irregularly triangular or kidney-shaped, surrounded by the wing (Fig. 1a), and originate from anatropous, crassinucellate, bitegmic ovules. The main part of the seed consists of endosperm (Fig. 1c and d). On the abaxial side of the seed, there are remnants of the funiculus,

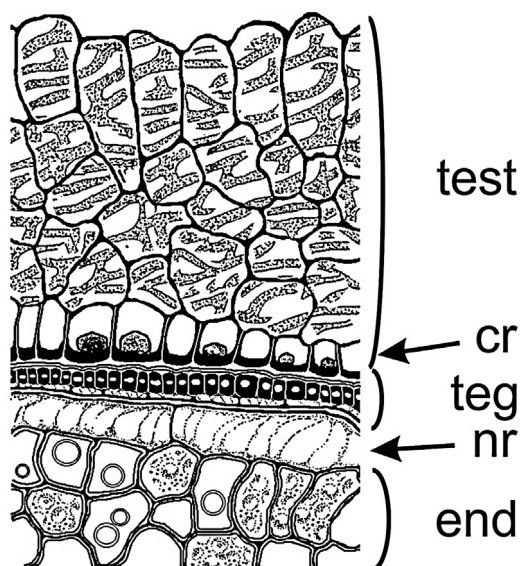
**Table 1**  
Seed and embryo characters and seed set in *Aristolochia contorta*.

Character	Means ± SE	Min.	Max.	CV <sup>b</sup> (%)
Seed length (mm)	6.67 ± 0.06	4.31	8.48	13.7
Seed width (mm)	10.02 ± 0.10	7.23	13.04	14.0
Seed length minus wing (mm)	3.59 ± 0.04	2.25	4.90	14.7
Seed width minus wing (mm)	5.60 ± 0.02	2.37	8.02	16.2
Seed surface area <sup>a</sup> (mm) <sup>2</sup>	67.63 ± 1.14	35.42	108.75	24.7
Seed mass, g per 100 viable seeds	1.13 ± 0.02	1.1	1.2	3.6
Seed mass, g per 100 aborted seeds	0.17 ± 0.03	0.12	0.21	27.1
Embryo length (μm)	853.35 ± 35.66	648.11	1002.7	12.5
Embryo width (μm)	290.77 ± 14.74	248.47	365.34	16.0
Embryo thickness (μm)	53.55 ± 2.98	33.0	64.0	16.7
Cotyledon length (μm)	379.32 ± 18.67	270.25	419.97	14.8
Viable seeds per fruit	48.50 ± 6.08	2	131	61.5
Aborted seeds per fruit	133.58 ± 7.44	61	188	27.3
Ovules per fruit	182.08 ± 5.76	123	256	15.5
Seed set (%)	26.7 ± 3.4	1.20	65.5	59.9

<sup>a</sup> The seed surface area is the product of the length and width (one side only).

<sup>b</sup> CV – coefficients of variation.





**Fig. 2.** The seed coat of *Aristolochia contorta*: test – testa, cr – crystalline layer, teg – tegmen, nr – the remnants of the nucellus, end – endosperm.

which are easily separated (Fig. 1a, below; 1d). The mature seeds of *A. contorta* have a thick seed coat (Figs. 1d and 2), and the number of cell layers of the testa varies from 6 to 8 on the adaxial side and 3–5 layers on the abaxial. The testa cells have a reticulate thickening (Fig. 2, test). The inner epidermis consists of a crystalline layer, which has thickened cell walls on the side near the tegmen. Two layers of crystalline cells are often observed near the seed wings. The tegmen consists of 4 layers. The outer side adjacent to the testa is formed by two layers of fibrous cells lying crosswise to each other, and the fibrous cells have thick lignified walls enclosing narrow intracellular space (Fig. 2, teg). Under this layer, there are remnants of sieve cells and a very thin fibrous layer that stained with hematoxylin. We observed remnants of the nucellus, with only the cell walls between the endosperm and the fibrous layer (Fig. 2, nr). A cavity is present in all of the mature filled seeds, with remnants of the hypostase between the tegmen and testa in the area opposite to the embryo (Fig. 1c).

### Embryo structure

The embryos are small, flat, and linear; they fill one-fifth of the mature seed and are located in the basal part of the seed. The embryo locule is skewed toward the middle part of the seed (Fig. 1d). The embryos have obvious cotyledons and a hypocotyl that is longer than the cotyledons (Fig. 3). The embryo root has 7–10 layers of cells and a functionally active suspensor. The apical meristem is flat. The cotyledons are usually combined with each other and lie in parallel; in some cases, one of the cotyledons is shifted perpendicular to the axis of the embryo. Five veins are formed in each of the cotyledons in the mature embryo. Occasionally the endosperm is present between the cotyledons: in this case, the endosperm cells near the epidermis of the cotyledons are swollen, loosened and broken, indicating their supply function for the cotyledons (Fig. 3). The endosperm is cellular, and the cells contain lipid droplets and starch. The quantitative parameters of the embryo are given in Table 1.

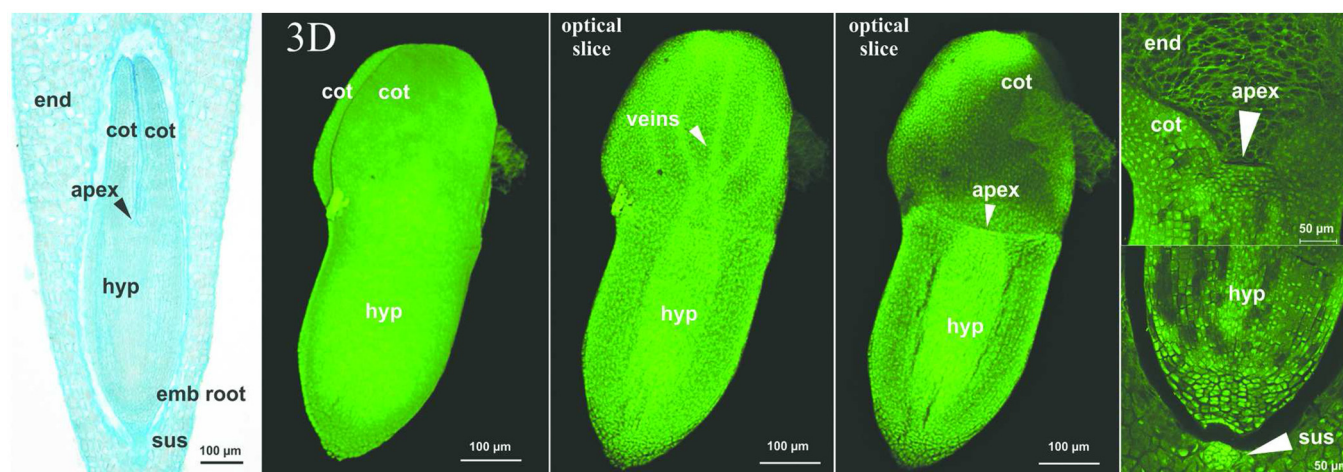
### Seed production

The parameters of reproductive success were highly variable. The number of viable seeds and seed set were more variable than the other seed traits (Table 1). The number of ovules per fruit exhibits a moderate variation level (CV = 15.5%), but it varied twofold between fruits with extreme values. The number of viable seeds varied greatly, from 2 to 131, with an average value of 48.5. The seed production efficiency, expressed as the seed set, was rather low (Table 1), i.e., only approximately a quarter of the ovules developed into normal seeds.

### Discussion

#### Seed size

Seeds of the genus *Aristolochia* are described as typically flattened, with or without a wing, and adapted to at least two different dispersal mechanisms, anemochory and zoochory (González and Stevenson, 2002). As it was shown for other *Aristolochia* species, the seeds with wings are much lighter than the wingless seeds. The mean weight of a single seed of *A. contorta* is 11.3 mg (Table 1) and 7.2 mg for *A. galeata* (Alves-da-Silva et al., 2011), whereas the values range between 23 and 28 mg for the wingless seeds



**Fig. 3.** Embryo structure of *Aristolochia contorta*, showing a longitudinal section of the embryo and different projections: end – endosperm, apex – apical meristem, cot – cotyledons, hyp – hypocotyl, emb root – embryo root, sus – suspensor, veins.

of *A. macrophylla*, *A. tomentosa*, *A. californica*, and *A. manshuriensis* (Adams et al., 2005a). The surface area of winged *A. contorta* seeds (Table 1) varies greatly, with a mean value ( $67.6 \text{ mm}^2$ ) similar to that of a wingless *A. macrophylla* seed (approximately  $66 \text{ mm}^2$ ) and the approximately  $62 \text{ mm}^2$  of an *A. manshuriensis* seed (Adams et al., 2005a). The reduction of the size found in a portion of the viable seeds (Table 1), the correspondingly lower weight, and the presence of an air cavity in the *A. contorta* seed may be an adaptive strategy. This contributes also increasing the number of seeds per capsule. Furthermore, the small seeds may be more efficient propagation units by anemochory and hydrochory over long distances. The seed-size variability may also reflect developmental or resource constraints.

### Seed structure

The seed traits of *A. contorta*, especially the presence of the wing, are similar to those of some other *Aristolochia* species – *A. acuminata*, *A. indica*, *A. petersiana* (Huber, 1985), species of Old World section *Diplolobus*, which are in the same clade along with *A. contorta* (González and Stevenson, 2002), – and also the representatives of the New World section *Gymnolobus*, *A. galeata* (Alves-da-Silva et al., 2011) and species from series *Thysicae* of the same section (González and Rudall, 2003; González and Stevenson, 2002). Seed wings seem to be independently derived in several major clades of this genus (González and Stevenson, 2002) as adaptation to wind and wind/water dispersal. It is interesting that Huber (1985) noted the similarity in the wing structure for the seed of *A. contorta* and the representative of the section *Gymnolobus*, *A. grandiflora*. It can be assumed that the seed wing is formed by an extension of either the seed or the funiculus, or of both (González and Rudall, 2003). Yet, valid comparison of wing structure in different species requires careful examination of the ontogeny and histology of the wing. Similar to the seeds of *A. contorta*, seeds of *A. maxima* show also a fusion of the funiculus in the central part. The seed coat structure is typical for *Aristolochiaceae*. The cells of the inner layer of the testa have crystals, and the outer and inner layers of the inner integument are parallel to the seed axis, whereas the middle layer is transversely oriented, forming cross fibers (González and Rudall, 2003; González and Stevenson, 2002; Huber, 1985; Mohana Rao, 1989). It should be noted that the walls of crystalline cells in the *A. contorta* seed were described earlier as “thin” (Huber, 1985), whereas in our work it was found that these cells have thickened cell walls on the side near the tegmen (Fig. 2, cr). The layer of the crushed cells between the inner integument and the endosperm (the remnants of the nucellus) is also characteristic of the family (Huber, 1985). The main difference from the seed coats of other *Aristolochia* species is the increase in the number of cell layers in the testa.

### Embryo structure

The embryo of *A. contorta* is well differentiated and small in size compared to the seed length minus the wing (ratio approximately 0.20). Based on the classification of Baskin and Baskin (1998) the embryo of *A. contorta* can be described as underdeveloped: It is differentiated, i.e., it has a discernible axis and two cotyledons, but it does not fill one-half of the seed length. A small underdeveloped embryo was described also for the previously studied *Aristolochia* species: *A. macrophylla* (embryo length to seed length ratio of 0.23); *A. tomentosa* (0.26); *A. californica* (0.38); *A. manshuriensis* (0.21) – Adams et al., 2005a,b, 2011, and *A. galeata* (0.07) – Alves-da-Silva et al., 2011). It is known that *Aristolochia* seeds with underdeveloped embryos require different periods of time

at suitable temperatures to germinate (Adams et al., 2005b, 2011; Alves-da-Silva et al., 2011). This also appears to be necessary for *A. contorta* seeds. Obviously, the preservation of a functionally active suspensor of the embryo plays an important role in the maturation of the embryo.

### Seed production

The number of seeds per capsule varies considerably in *Aristolochia* species: *A. paucinervis*/11 seeds (Berjano et al., 2006); *A. baetica*/35–45 seeds (Berjano et al., 2011); *A. manshuriensis*/145 seeds (Nakonechnaya et al., 2005); *A. macrophylla*/90–93 seeds; *A. tomentosa*/198–209 seeds; *A. californica*/85–91 seeds (Adams et al., 2005a); *A. inflata*/140 seeds, and *A. maxima*/520 seeds (Sakai, 2002). There is scarce information about the seed set of *Aristolochia* species: our value for *A. contorta* was similar to that of *A. paucinervis* (25.6%) and is considerably lower than that of *A. baetica* (approximately 50%) – our own calculations are based on the data of Berjano et al. (2006, 2011) – and *A. manshuriensis* (95.5%; Nakonechnaya et al., 2005). Our finding is also lower than the seed/ovule ratio (48%) known for outcrossers (Wiens et al., 1987). It is known that the failure to set seed can be attributed to a number of factors, including effects of climatic factors, pollinator limitations and pollen quality, resource limitations and genetic factors (inbreeding depression and genetic load (Charleworth, 1989; Helenurm and Schaal, 1996; Wiens et al., 1987). Some of these factors are considered as the main causes of the low fruit set in *A. paucinervis* (Berjano et al., 2006), *A. baetica* (Berjano et al., 2011), and in *A. manshuriensis* (Nakonechnaya et al., 2005). In the natural habitats of *A. contorta*, the climatic conditions during flowering (low temperatures and high humidity) can result in pollinator limitation. The high proportion of defective pollen grains in *A. contorta* (24.5%; Nakonechnaya et al., 2007) may also restrict pollination and constrain fruit initiation. The flowers of *A. contorta* produced a mean of 182 ovules (Table 1). It is possible that the plant is not able to achieve the full maturity of so many seeds and the high rate of seed abortion seems to be a consequence of resource limitations. The competition for resources among ovaries can be responsible for the abortion of the seeds in multi-seeded fruits (Helenurm and Schaal, 1996). The restricted seed production of *A. contorta* may also be related to genetic factors. Reduced productivity in predominantly outcrossing species is generally attributed to genetic load associated with high levels of heterozygosity (Charleworth, 1989; Wiens et al., 1987). There are reportedly moderate levels of heterozygosity ( $H_o = 0.118–0.144$ ) and an excess of heterozygotes ( $F_{IS} = -0.282$ ) in the *A. contorta* populations studied (Nakonechnaya et al., 2012). It is possible that the low seed set in the outcrossing perennial *A. contorta* can be attributed to genetic load, including both segregational and mutational loads. The factors listed above are not mutually exclusive, and each of them may contribute to the relatively low seed yield in *A. contorta*. Given the late start of flowering (mid-June) and the short period of vegetative growth (approximately 4 months), *A. contorta* on the northern edge of its range does not have sufficient time to form a complete seed stock, which further limits the success of seedling maintenance.

This study examined *A. contorta* and documented for the first time certain seed and embryo traits that were found to be variable and may be potentially informative at both the genus and species levels. The presence of a wing and the seed-size variation may promote the successful dissemination of *A. contorta*. The structure of the seed coat does not prevent seed germination. However, the restricted productivity and reduced population size are threats to the continued survival of this species.

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