Reviewing the identity of the Maltese *Polypodium* (Polypodiaceae) – new evidence from morphology and flow cytometry

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**Abstract**

The first record of *Polypodium* from Gozo (Maltese Islands) was described as a new endemic taxon, *Polypodium vulgare* subsp. *melitense*, based on its unique set of morphological characters. It was treated as a novelty and designated as a subspecies of *P. vulgare* mainly due to the lack of paraphyses, the presence of 10–16 annular cells, and a mean spore length of 64 µm. The fern was reassessed by us employing a more rigid morphological analysis and the application of flow cytometry. The absence of paraphyses was confirmed, but the number of annular cells (5–11) and the spore length (70–79 µm) differed from the previous study. These and other morphological traits, the phenology (leaf-shedding in spring), the calcareous growth habitat, and the southern distribution implied that the fern is *P. cambricum.* Final confirmation was obtained from flow cytometry; the genome size of 17 pg corresponds perfectly with the range obtained for other accessions of this diploid species. The *Polypodium* reported from Malta does not merit taxonomic distinction despite being a rare form of *P. cambricum*, which lacks paraphyses.

**Keywords**: *Polypodium vulgare subsp. melitense*, nuclear DNA amount, paraphyses, ferns, Flora of Malta, Central Mediterranean Region

**Introduction**

In Europe (excluding Macaronesia), the genus *Polypodium* L. is represented by three species, the diploid *P. cambricum* L. (2*n* = 74), the tetraploid *P. vulgare* L. (2*n* = 148) and the hexaploid *P. interjectum* Shivas (2*n* = 222). Interspecific hybrids between these are well documented and comprise *P*.×*font-queri* Rothm. (*P. cambricum* × *P. vulgare,* 2*n* = 111), *P*. ×*shivasiae* Rothm. (*P. cambricum* × *P. interjectum*, 2*n* = 148), and *P*. ×*mantoniae* Rothm. (*P. interjectum* × *P. vulgare,* 2*n* = 185).

Other diploid *Polypodium* taxa occur in the Macaronesian Islands (Azores, Canaries, and Madeira). Previously, these were commonly treated as subspecies of *P. cambricum*, such as *P. cambricum* subsp. *azoricum* (Vasc.) E. Nardi restricted to the Azores and *P. cambricum* subsp. *macaronesicum* (A.E. Bobrov) Fraser-Jenk. endemic to Madeira and the Canaries. Using chloroplast DNA data, Rumsey et al. (2014) recognized *P*. *macaronesicum* A.E. Bobrov as a distinct taxon with two subspecies, one Azorean and one Madeiran–Canarian. Results recently obtained by flow cytometry (Fuchs et al., unpublished) show, however, that the plants from the Azores appear clearly separated from all other diploids and well merit specific rank suggesting that the name *P*. *azoricum* (Vasc.) R. Fernandes should be used to acknowledge the endemic status of this taxon (Schäfer 2001, 2005). The triploid hybrid (2*n* = 111) *P*.×*encumeadense* (Neuroth, Jäger & Bennert) F.J. Rumsey, Carine & Robba (*P. macaronesicum* s. str. × *P. vulgare*) has been described from Madeira (Neuroth et al. 1998).

A *Polypodium* taxon believed to be endemic to the Maltese Islands was reported by Peroni et al. (2013) based on the discovery of two small populations in southern parts of Gozo (by author SM). The population found at the limits of Ta’ Ċenċ at the locality of Ta’ Sannat, consisted of one individual growing in a small crevice of karst rock composed of upper coralline limestone, approximately 150 m above sea level (Fig.1). The other population, also of one individual, was found in a small, sub-vertical rock basin in the same type of rock situated at the very edge of the vertical escarpments of Wied il–Kbir, in San Lawrenz, approximately 80 m above sea level. The plant at Ta’ Ċenċ was medium-sized and had 15 fronds, while the other one at Wied il–Kbir was much smaller having only three small leaves. Failing to identify the fern using standard determination keys (Pignatti 1982, Muñoz Garmedia 1986, Valentine & Moore 1993), Mifsud submitted the material to the pteridologists Dr. Adalberto Peroni and Dr. Gilberto Peroni (Varese, Italy). They found the Maltese fern unique and opted to describe the fern as a new taxon, which was named *P*. *vulgare* subspecies *melitense* Peroni, Peroni & Mifsud (Peroni et al. 2013).

The description of *P*. *vulgare* subspecies *melitense* was based only on morphological biometrics, and no molecular analyses were carried out. This appeared to be justified, because the set of macro- and micro-morphological characters of the Maltese polypody did not match those of any of the described European *Polypodium* taxa. Hybrids, which produce aborted spores, were ruled out due to the well-developed spores of the Maltese plants. Due to rounded sori lacking paraphyses and the sporangia having a high number of indurated cells (10–16), the Peroni brothers ascribed the Maltese fern to *P. vulgare*. Hence, assuming that the paraphyses and indurated cells in the annulus have the highest taxonomic value (Roberts 1970, Nardi & Tomei 1976). However, the plants also exhibit several characters diagnostic on *P. cambricum*, including the size and the shape of the fronds, their deciduous habit, their calcareous substrate (*P. vulgare* prefers acidic rock; Page 1997), and rhizome scales 4–10 mm in length (Roberts 1970; Hutchinson & Thomas 1996). Furthermore, *P. vulgare* has a mainly northern distribution, and does not occur in Sicily (Pignatti 1982; Pignatti et al. 2017; Giardina et al. 2007). Also, *P. vulgare* is a weak calcifuge, which prefers to grow in various types of acidic rock (Page 1997) and growth on calcareous rocks is not to be expected. Given an apparent affinity of *P*. *vulgare* subspecies *melitense* to *P. cambricum*, a reassessment of the morphology and he genome size of this Maltese fern was carried out in this study. This reassessment is deemed important to clarify the taxonomic status of this taxon which is currently considered as a Maltese endemic. Its genome size is compared with those of *P*. *cambricum, P. interjectum*,and *P. vulgare* from Europe.

**Material and methods**

Macro- and micromorphological analyses

The type locality of *P*. *vulgare* subsp. *melitense* was revisited at Ta’ Ċenċ repeatedly (13 January 2015, 8January 2017, and 24 January 2019). The number of fronds were counted to monitor the growth. Measurements and morphological examinations of the fronds were carried out *in situ*. The small fern at San Lawrenz was not studied because of the difficulty to access it and for conservation purposes (one individual with just three small fronds when found in 2012). Three pinnae with mature sori were sampled for microscopical examination of the sporangia and their spores. Another small sample was collected for determination the nuclear genome size. Special attention was paid to the presence of paraphyses in the sori. Investigations of the stomata were omitted, because their size stomata size overlaps in three *Polypodium* species and thus are not taxonomically diagnostic.

The total length of the fronds includes the petiole. For calculating the length to width ratio of the lamina, the petiole was excluded, and the widest part of the lamina of largest three fronds was chosen. Young and mature sori were examined with a stereomicroscope (×10, ×40) screening for the presence of paraphyses. Sporangia and spores were detached from five sori using a stainless steel needle and transferred into a drop of water on a microscope slide. It was then covered with a coverslip, pressed gently to squash and force some sporangia to open and mounted under a light microscope. The number of basal cells (not reported by Peroni et al. 2013) and indurated cells were counted for 40 sporangia. Spores were photographed against an eyepiece–mounted reticule, previously calibrated with a scale micrometer. The length and width of 31 spores were measured from the micro-photographs using the software Pixmetre by Par Alain Henriot. Studies were carried out from the individual at Ta’ Ċenċ. The values of the Maltese *Polypodium* were compared with literature data for *P. vulgare, P. interjectum*, and *P. cambricum* from different regions (Italy, Nardi & Tommei, 1975; Spain, Muñoz Garmedia 1986; and Great Britain, Page 1997).

Flow cytometry

For flow cytometric studies, fresh plant material was used from cultivated plants of *Polypodium* collected in Finland, France, Germany, Great Britain, Ireland, Italy, Malta, Spain, and Turkey. Nuclear DNA content was estimated using roughly 0.5 cm2 of pinna tissue of from each individuals mixed with equivalent amounts of fresh leaf tissue of *Pisum sativum* L. subsp. *sativum* convar. *sativum* var. *ponderosum* Alef., Sorte Viktoria, Kifejtö Borsó (IPK Genebank accession number: PIS 630; 9,07 pg/2C) as an internal reference standard. Nuclei were isolated as described previously (Dolezel et al. 2007) using the nuclei isolation buffer according to Galbraith et al. (1983) supplemented with 1% PVP-25, 0.1% Triton x-100, DNase-free RNase (50µg/ml) and propidium iodide (50µg/ml). For every accession, four independent isolations were measured using a CyFlow Space flow cytometer (Sysmex-Partec). The absolute DNA content (pg/2C) were calculated based on the values of the G1 peak means.

**Results**

Morphology

The morphological characters of the Maltese specimen are summarised in Table 1, where they are compared with those of *P. cambricum, P. interjectum,* and *P. vulgare*. In 2015, the plant from Malta had nine fronds (the longest measuring 11.5 cm and having 21 pinnae); in 2017 (Fig. 1C), it had seven fronds (the longest was 10.0 cm and had 17 pinnae), whereas in 2019, the same plant had ten fronds (with the longest measuring 13.5 cm and having 23 pinnae). As for the sporangia (Fig. 1A), the number of basal cells was 3 to 4 (these were not taken into account by Peroni et al. 2013) and the number of indurated cells varied between 5 and 11 instead of 10 to 16 as reported by Peroni et al. 2013. However, concurring with this earlier study, the lack of paraphyses was also confirmed in our reassessment.

Nuclear genome size

For all three species, a number of plants from multiple European localities were analysed (see Table 2). Within each species, no obvious geographical variability exists; all differences between localities are small, likely due to measurement errors of the method applied. In accordance with the ploidy level and the chromosome number, the nuclear genome size varies greatly between species. With a mean of 17.28 pg, the 2C DNA amount is smallest in the diploid *P. cambricum*, followed by the tetraploid *P. vulgare* (29.41 pg) and by the hexaploid *P. interjectum* (45.10 pg). The genome size of the plant from Malta is 17.09 pg, which suggests that it is diploid, thus representing *P. cambricum*.

The *P. vulgare* complex has repeatedly been studied by methods quantifying the DNA amount. For various reasons, older determinations (like those using Feulgen microdensitometry or flow cytometry applying ethidium bromide as a nucleic acid stain) yielded deviating results, as Bureš et al. (2003) have shown. These authors studied *Polypodium* taxa in the Czech Republic using propidium iodide like we did. They report DNA amounts very similar to ours: 29.00 pg for *P. vulgare* and 45.24 pg for *P. interjectum*; they did not investigate *P. cambricum* as it does not occur in the Czech Republic. *Polypodium* hybrids (mainly *P*. ×*mantoniae*, the cross between *P. vulgare* and *P. interjectum*) have also has been investigated repeatedly and were shown to have DNA amounts intermediate to those of their parents (Bureš et al. 2003, Helánová et al. 2004, Szczȩśniak et al. 2015).

**Discussion**

The results reported here are significantly different from that concluded by Peroni et al. (2013). Almost all morphological characters as well the phenology, habitat, and distribution suggest that the Maltese *Polypodium* is *P. cambricum*, rather than *P. vulgare*. One character that differentiates the three European *Polypodium* species is the phenology. Only *P. cambricum* sheds the leaves in spring, remains dormant throughout summer forming new fronds in autumn (usually after the first rains), and produces sori by end of autumn that mature in early winter. Moreover, the number of indurated cells in the annulus of the sporangium, the number of its basal cells, and the spore size are important morphological characters, and match perfectly with *P. cambricum* (Table 1). It is surprising that the number of indurated cells found in this study (5–11, Fig. 1B) differs from the number reported by Peroni et al. (2013), who claim to have counted 10–16 indurated cells. Concurring with their study, we could not detect any paraphyses, normally a good character to distinguish *P. vulgare* (paraphyses absent) from *P. cambricum* (paraphyses present). Notably, paraphysis-free specimens of *P*. *cambricum* have been found in France and Minorca (pers. comm. with Errol Vela and Pere Fraga, respectively; Jan 2019). Conversely, Neuroth (1996) detected paraphyses in a few specimens of *P*. *vulgare* and *P*. *interjectum*, indicating that exceptions occur both ways. The genome size provides clear evidence that the Maltese specimens are indeed *P. cambricum*—

With 17.09 pg it is fully in the range of the diploid species and matches perfectly with those of *P. cambricum* from other European localities (Table 2).

Creating a taxon at the variety level to accommodate the Maltese plants of *P. cambricum* without paraphyses (it could be named *P. cambricum* var. *melitense* nom prov.) might be considered. However, since there is no obvious correlation of the paraphysis-free character with altitude, habitat, geographic distribution (their occurrence seems to be sporadic), and there is no other supporting characteristic in common, describing a new variety does not seem to be appropriate. Paraphyses are easily overlooked, as they deteriorate or degenerate in mature sori (Shivas 1962). For this reason, both young and mature sori were examined in this study. A study of why some plants of *P. cambricum* do not form paraphyses and whether there is any pattern of their presence/absence throughout its geographical range may illuminate patterns of distribution of this character but is beyond the scope of this study.

**Conclusions**

The reassessment of the morphological characters of the *Polypodium* species occurring in the island of Gozo (Maltese Islands), as well as the habitat, phenology, geographic distribution, genome size, and ploidy level, confirms that it corresponds to *Polypodium cambricum*, and the taxon *P. vulgare* subsp. *melitense* is therefore its synonym. The native ferns and lycophytes occurring on the Maltese islands and their frequency as estimated in the last 20 years are:

1. *Selaginella denticulata* (L.) Spring (scarce-rare)

2. *Equisetum ramosissimum* Desf. (frequent in some localities)

3. *Adiantum capillus-veneris* L. (frequent)

4. *Anogramma leptophylla* (L.) Link (frequent in some localities)

5. *Asplenium sagittatum* (DC) Bange (rare)

6. *Asplenium ceterach* L. (rare)

7. *Asplenium trichomanes* L. (rare)

8. *Asplenium marinum* L. (very rare)

9. *Pteridium aquilinum* (L.) Kuhn (very rare)

10. *Polypodium cambricum* L. (very rare, see Fig. 1)

*Isoëtes histrix* Bory var. *subinermis* Durieu, *Salvinia natans* (L.) All., *Equisetum fluviatile* L., *Equisetum arvense* L., and *Scolopendrium vulgare* Sm. were recorded in historic literature (Gulia 1909). Since not reconfirmed for about a century, they are presumably extinct. Some historic records have been misidentified, such as the records of *Scolopendrium vulgare* which refer to *Asplenium sagittatum* (Mifsud et al. 2016), and the occurrence of *Equisetum fluviatile* and *E. arvense* seems improbable and considered as misidentifications of *E. ramosissimum* (Sommier and Caruana-Gatto 1915). A few other historic records might have been casual introductions, for example *S. natans*, which was sighted by Gulia (1909) on ponds at Wied il-Lunzjata, Gozo, but not confirmed few years later, and whose presence in Malta was consequently doubted by Sommier and Caruana-Gatto (1915).

Nevertheless, the Maltese islands has a rather high richness of ferns when compared to other sedimentary islands in the central Mediterranean region (Troia et al. 2012), but the species are regressing and becoming extinct, presumably due to a warmer and drier climate change, habitat loss and pressures from anthropogenic activities.

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| **Characteristics and**  **observations** | *Polypodium* from Malta | *Polypodium cambricum* | *Polypodium interjectum* | *Polypodium vulgare* |
| Development of new fronds | October | Autumn | Spring, autumn | Spring, early summer |
| Spore maturation | December | Autumn | End of spring to early summer | Spring |
| Frond life span | Fronds die off in April | Fronds die off in spring | Fronds remain green until next summer | Fronds remain green until next summer |
| Habitat | Shaded cavities in calcareous rocks | Calcareous cliffs, walls, bark of trees | Wide range of rock types (often on calcareous cliffs), walls, bark of trees | Cliffs (often acid), walls, bark of trees |
| Length of rhizome scales | 4–10 mm | 5–16 mm (es) | 3.5–10 mm (es) | 3–6 mm (es) |
| Maximal frond length | 13.5 cm | 57 cm (es)  40 cm (uk) | 53 cm (es)  60 cm (uk) | 47 cm (es)  25 cm (uk) |
| Length to width ratio of fronds | 1.4–1.5 | 0.8–2.5 (es)  1.6 (it) | 1.2–3.7 (es)  2.6 (it) | 1.4–3.4 (es)  2.9 (it) |
| Shape of leaf lamina | Broadly oblong-ovate to triangular-ovate | Oblong-ovate to triangular-ovate | Oblong-lanceolate to subdeltoid. | Narrowly oblong-lanceolate to ovate-lanceolate |
| Secondary veins of pinnae | 1–2 times dichotomous | 3–4(–6) times dichotomous | 3–4 times dichotomous | 1–3 times dichotomous |
| Shape of young sori | Round to sub-elliptical | Elliptical | Elliptical | Round |
| Paraphysis | Absent | Present 400– 1800 µm long (es) | Absent | Absent |
| No. of indurated cells | 5–11 (Fig. 1A) | (2)5–12(18) (es)  (5)6–8(11) (it)  4–19 (uk) | (4)7–10(13) (es)  6–9(11) (it)  4–13 (uk) | (7)11–14(18) (es)  (8)10–13(14) (it)  7–17 (uk) |
| No. of basal cells | 3-4(5) (Fig. 1A) | (2)3–4(5) (es)  (2)3–4 (it)  3–4 (uk) | (1)2–3(4) (es)  2–4 (it)  2–3 (uk) | (0)1(2) (es)  (0)1 (it)  1 (uk) |
| Spore length (μm) | (68)70 –79(80) | (60)62–74(78) (es) | (70)74–88(90) (es) | (54)56–68(72) (es) |
| Mean spore length (μm) | 74.7 | 68 (es)  76 (it) | 81 (es)  87 (it) | 61 (es)  60 (it) |
| Spore L/W ratio | 1.3–1.7  (mean 1.5) | Mean 1.5 (it) | Mean 1.6 (it) | Mean 1.4 (it) |
| Ploidy (and chromosome number) | Diploid | Diploid  (2*n* = 74) | Hexaploid  (2*n* = 222) | Tetraploid  (2*n* = 148) |
| Occurrence in Sicily (Giardina et al. 2007) | – | Yes, common | Yes, rare | No |

Table 1. Characteristics of *Polypodium* sp. from Ta’ Ċenċ, Sannat, Gozo compared with those of *P. cambricum, P. interjectum,* and *P. vulgare* as reported from Italy (it) by Nardi & Tommei (1976); from Spain (es) by Muñoz Garmedia (1986), and from Great Britain (uk) by Page (1997).

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| --- | --- | --- | --- |
| **Species** | **Locality** | **Collector** | **DNA 2C [pg] mean ± SD** |
| ***P. cambricum*** | France, Corsica, Cap Corse | WB | 17.27 ± 0,25 |
|  | France, Departement Vaucluse | HT | 17.09 ± 0,04 |
|  | Great Britain, Wales, Preserved County Clwyd, Llandudno | MR | 17.26 ± 0,12 |
|  | Ireland, County Sligo, Loch Gill | MR | 17.45 ± 0,15 |
|  | Italy, Provincia di Siracusa, Sicily, Sortino | HT | 17.16 ± 0,07 |
|  | Italy, Provincia di Livorno, Capraia Isola, Porto Vecchio | WB | 17.41 ± 0,11 |
|  | Spain, Mallorca, Biniaraix | WB | 17.32 ± 0,14 |
|  | **Mean of localities** |  | **17.28 ± 0,13** |
|  | Malta, Ta’ Ċenċ, Gozo | SM | 17.09 ± 0,04 |
|  |  |  |  |
| ***P. interjectum*** | France, Corsica, Cap Corse, Col Saint Jean | WB | 45.39 ± 0.27 |
|  | France, Corsica, Corte | WB | 45.14 ± 0.46 |
|  | Germany, Rhineland-Palatinate, Bad Münster am Stein | WB | 45.26 ± 0.80 |
|  | Germany, North Rhine-Westphalia, Hönnetal | WB | 45.82 ± 0.14 |
|  | Great Britain, Scotland, Port Logan | MR | 45.08 ± 0.82 |
|  | Italy, Isola d’Elba, Monte Capanne | WB | 44.68 ± 0.45 |
|  | Turkey, Kaz Dağı, Edremit | WB | 44.47 ± 0.47 |
|  | **Mean of localities** |  | **45.10 ± 0,49** |
|  |  |  |  |
| ***P. vulgare*** | Finland, Larsmo, Köpmanholmen | SM | 29.13 ± 0.22 |
|  | Finland, Larsmo, Krok | SM | 29.28 ± 0.09 |
|  | France, Dépt. Loire, Saint George en Couzan | JCB | 29.43 ± 0.14 |
|  | Germany, Rhineland-Palatinate, Norheim | WB | 29.97 ± 0.18 |
|  | Germany, Rhineland-Palatinate, Schloss Dhaun | WB | 29.22 ± 0.13 |
|  | **Mean of localities** |  | **29.41 ± 0,33** |

Table. 2. Nuclear DNA content of the three *Polypodium* species as analysed for various European localities; the Maltese taxon is separately listed under *P*. *cambricum*; for each species mean and standard deviation is indicated. Abbreviations used for collectors: HT = Hjalmar Thiel (Jameln, Germany), JCB = Jean-Charles Bertier (Montbrison, France), MR = Martin Rickard (Tenbury Wells, Great Britain), SM = Stephen Mifsud (Gozo, Malta), WB = H. Wilfried Bennert (Ennepetal, Germany).

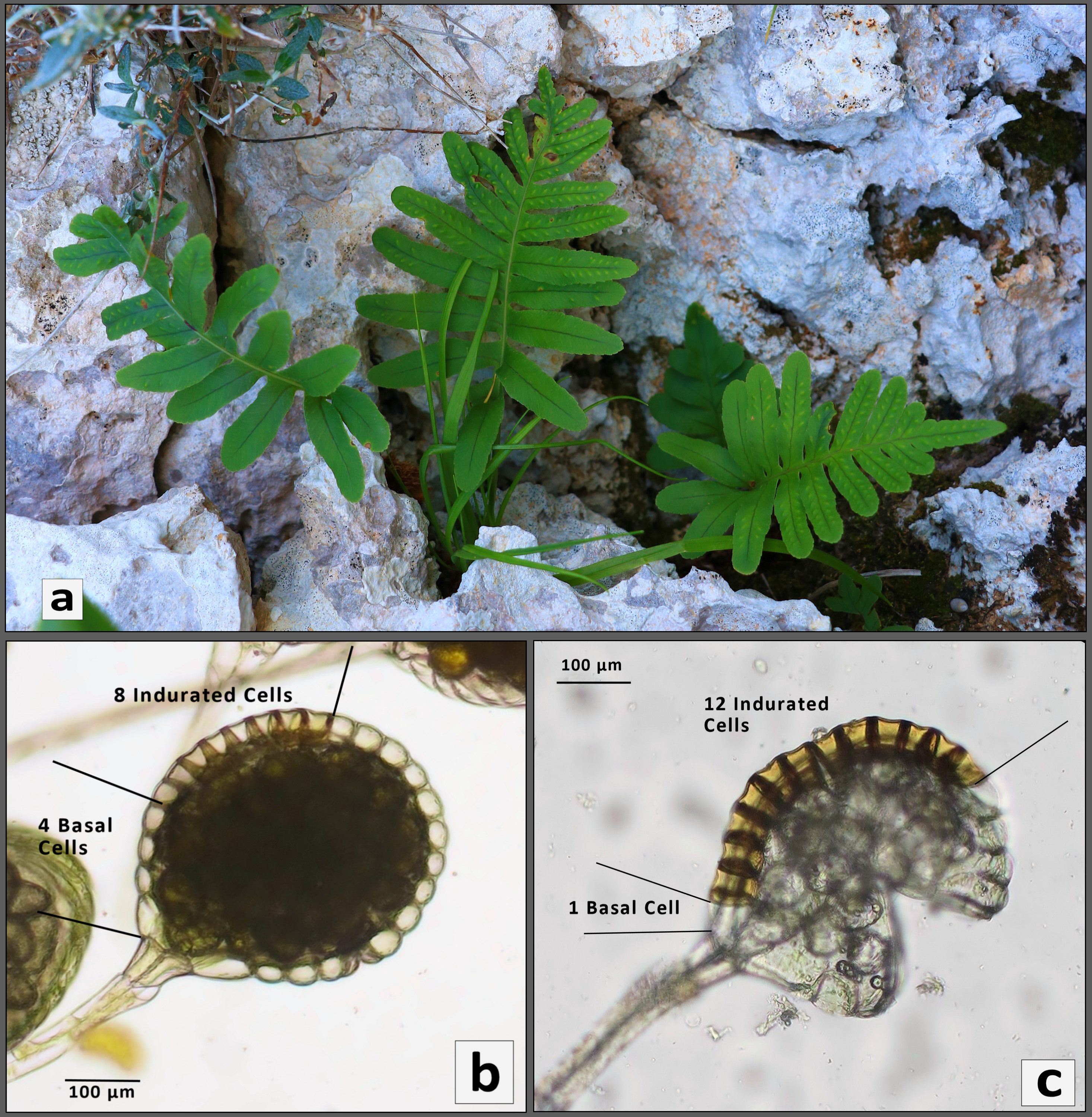


Figure 1. Habit of Polypodium cambricum at Ta’ Ċenċ, Sannat, Gozo, Malta (a), and sporangia of this plant (b) compared with a sporangium of P. vulgare (from a plant growing near Köpmanholmen, Larsmo, Finland; c).