KAREN E. JAMES

19. DNA BARCODING DARWIN'S MEADOW

A Twenty-first-century Botanical Inventory at Historic Down House

CHARLES DARWIN'S 1855 SURVEY OF A MEADOW NEAR DOWN HOUSE

> If ever you catch quite a beginner, and want to give him a taste for Botany, tell him to make a perfect list of some little field or wood.. . it gives a really uncommon interest to the work, having a nice little definite world to work on, instead of the awful abyss and immensity of all British Plants.[1]

In June 1855 Charles Darwin wrote to Joseph Hooker that he intended to survey the 13-acre hay meadow (Great Pucklands) near Down House, and reported with delight – 'Hurrah! Hurrah!' – that he had identified his first grass.[2] He and Miss Thorley, his children's governess, began the survey that month[3] and by the end of that month had identified 28 species of grass[4]; by July, they were up to 35 species[5]. They were still conducting the survey in August when he wrote to John Henslow that he was puzzled by an umbellifer.[6]

Darwin's objectives in doing the survey were his and Thorley's 'amusement' (Darwin to Hooker, 5 June 1855) and 'to show the degree of diversity in our British plants on a small plot' (Darwin, 1857, p. 230); the survey was, perhaps, among the first intentional, comprehensive species counts in a geographically defined area in history. In an age when rare specimens were prized above all, their aim was radical: to identify all of the plant species growing on a small, unremarkable plot. He described the field and the result of the survey as follows:

> I selected a field, in Kent, of 13 acres, which had been thrown out of cultivation for 15 years, & had been thinly planted with small trees most of which had failed: the field all consisted of heavy very bad clay, but one side sloped & was drier: there was no water or marsh: 142 phanerogamic plants were here collected by a friend during the course of a year; these belonged to 108 genera, & to 32 orders out of the 86 orders into which the plants of Britain have been classed.

This narrative appeared in the second part of his original 'big species book' written from 1856 to 1858 (Darwin, 1857, p. 230), but about half-way through writing the book, on June 18, 1858 Darwin received the now famous letter from Alfred Russel Wallace which enclosed his February 1858 manuscript 'On the tendency of varieties

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to depart indefinitely from the original type'[7], which spurred him to write what he called 'an abstract' of the book: *On the origin of species*. In Chapter IV of that work, Darwin substituted his later count on a 3-foot by 4-foot patch of his lawn for the original Great Pucklands meadow survey (Darwin, 1859, p. 114; Randal Keynes, pers. comm.).

THE NATURAL HISTORY MUSEUM'S 2005-2007 RE-SURVEY OF THE MEADOW

The field that Darwin and Thorley surveyed, Great Pucklands meadow, is part of a larger area including Down House and the surrounding countryside (see Figure 1) that is the subject of a World Heritage Site bid '*Darwin's Landscape Laboratory*'.[8] During the summers of 2005, 2006 and 2007 a team from the Natural History Museum in London (NHM) re-surveyed Great Pucklands meadow (see Figure 2). As Darwin used Great Pucklands meadow in 1855 to develop his botanical identification skills – to get 'a taste for botany'[9] – so the team used the meadow to further develop procedures for plant identification 150 years later.

The aims of the re-survey were to (1) detect change (if any) in species number and diversity in the meadow since 1855, and (2) pilot and optimise procedures for high-throughput botany, pairing the collection and management of herbarium specimens together with 'DNA barcoding', the creation of libraries of short, standardized, DNA sequences linked to representative specimens in established specimen repositories, for the eventual use in DNA-based identification of unknown samples. Specifically,

Figure 1. Satellite image[10] (left) indicating the relative locations of Down House (top right) and Great Pucklands meadow (bottom right) on the south-west corner of Downe village in Kent. (Top right, bottom right © Karen James)

Figure 2. The NHM re-survey in 2007 of Great Pucklands meadow (top left) involved plant identification (top right), specimen collection (bottom left) and sampling for DNA barcoding (bottom right). (Top right, bottom left © Karen James; top left, bottom right: screen capture from the 2009 BBC programme, Jimmy Doherty in Darwin's Garden)

the NHM team aimed to evaluate a selection of short regions of DNA that had been identified as potential candidates for DNA barcoding (Chase et al., 2007; Kress & Erickson, 2007).

Though the DNA barcoding data from these specimens have been reported and analysed as part of a peer-reviewed, consortium-authored paper that recommended a 'DNA barcode for land plants' (CBOL Plant Working Group, 2009), that paper did not allow space to report or discuss the results of the re-survey, nor the DNA barcoding results in isolation from the larger consortium data set. This chapter provides an opportunity to discuss the importance of Charles Darwin's original survey of Great Pucklands meadow with the children's governess, Miss Thorley in relation to a twenty-first-century re-survey and the use of DNA barcoding in the context of a potential science learning environment.

The NHM team carried out preliminary surveys in 2005 and 2006, recording, but not collecting, 161 vascular plant species. In 2007 single specimens from 141 species – 138 flowering plants, two conifers and one fern (see Table 1) – were collected, along with digital photographs and Ordinance Survey National Grid references (not shown).

Table 1. List of seed plant species collected by the NHM team (2007) as per Stace (1997).

Order	*Family*	*Taxon*
Alismatales	Araceae	*Arum maculatum*
Alismatales	Araceae	*Lemna minor*
Apiales	Apiaceae	*Anthriscus sylvestris*
Apiales	Apiaceae	*Chaerophyllum temulum*
Apiales	Apiaceae	*Daucus carota*
Apiales	Apiaceae	*Heracleum sphondylium*
Apiales	Apiaceae	*Pimpinella saxifraga*
Apiales	Apiaceae	*Sanicula europea*
Apiales	Apiaceae	*Torilis japonica*
Apiales	Araliaceae	*Hedera helix*
Aquifoliales	Aquifoliaceae	*Ilex aquifolium*
Asparagales	Hyacinthaceae	*Hyacinthoides non-scripta*
Asterales	Asteraceae	*Achillea millefolium*
Asterales	Asteraceae	*Arctium minus*
Asterales	Asteraceae	*Centaurea nigra*
Asterales	Asteraceae	*Cirsium arvense*
Asterales	Asteraceae	*Hypochaeris radicata*
Asterales	Asteraceae	*Lapsana communis*
Asterales	Asteraceae	*Leucanthemum vulgare*
Asterales	Asteraceae	*Senecio erucifolius*
Asterales	Asteraceae	*Senecio jacobaea*
Asterales	Asteraceae	*Senecio vulgaris*
Asterales	Asteraceae	*Sonchus asper*
Asterales	Asteraceae	*Taraxacum officinale* agg
Asterales	Asteraceae	*Tragopogon pratensis*
Asterales	Asteraceae	*Crepis capillaris*
Asterales	Asteraceae	*Crepis vesicaria*
Brassicales	Brassicaceae	*Alliaria petiolata*
Brassicales	Brassicaceae	*Sisymbrium officinale*
Caryophyllales	Amaranthaceae	*Atriplex patula*
Caryophyllales	Caryophyllaceae	*Cerastium fontanum*
Caryophyllales	Caryophyllaceae	*Stellaria graminea*

(*Continued*)

Table 1. (Continued)

Order	*Family*	*Taxon*
Caryophyllales	Caryophyllaceae	*Stellaria holostea*
Caryophyllales	Polygonaceae	*Polygonum aviculare*
Caryophyllales	Polygonaceae	*Rumex acetosa*
Caryophyllales	Polygonaceae	*Rumex crispus*
Caryophyllales	Polygonaceae	*Rumex obtusifolius (x pratensis)*
Caryophyllales	Polygonaceae	*Rumex sanguineus*
Caryophyllales	Polygonaceae	*Rumex x pratensis*
Cornales	Cornaceae	*Cornus sanguinea*
Dioscoreales	Dioscoreaceae	*Tamus communis*
Dipsacales	Adoxaceae	*Adoxa moschatellina*
Dipsacales	Adoxaceae	*Sambucus nigra*
Ericales	Primulaceae	*Primula vulgaris*
Fabales	Fabaceae	*Lathyrus pratensis*
Fabales	Fabaceae	*Lotus corniculatus*
Fabales	Fabaceae	*Trifolium dubium*
Fabales	Fabaceae	*Trifolium pratense*
Fabales	Fabaceae	*Trifolium repens*
Fabales	Fabaceae	*Vicia sepium*
Fabales	Fabaceae	*Vicia tetrasperma*
Fagales	Betulaceae	*Carpinus betulus*
Fagales	Betulaceae	*Corylus avellana*
Fagales	Fagaceae	*Castanea sativa*
Fagales	Fagaceae	*Fagus sylvatica*
Fagales	Fagaceae	*Quercus ilex*
Fagales	Fagaceae	*Quercus robur*
Gentianales	Gentianaceae	*Centaurium erythraea*
Gentianales	Rubiaceae	*Galium mollugo*
Geraniales	Geraniaceae	*Geranium dissectum*
Geraniales	Geraniaceae	*Geranium molle*
Geraniales	Geraniaceae	*Geranium robertianum*
Lamiales	Lamiaceae	*Glechoma hederacea*

(*Continued*)

Table 1. (Continued)

Order	*Family*	*Taxon*
Lamiales	Lamiaceae	*Lamium album*
Lamiales	Lamiaceae	*Origanum vulgare*
Lamiales	Lamiaceae	*Prunella vulgaris*
Lamiales	Lamiaceae	*Stachys sylvatica*
Lamiales	Oleaceae	*Fraxinus excelsior*
Lamiales	Oleaceae	*Ligustrum vulgare*
Lamiales	Orobanchaceae	*Odontites vernus ssp. serotinus*
Lamiales	Plantaginaceae	*Plantago lanceolata*
Lamiales	Plantaginaceae	*Veronica chamaedrys*
Lamiales	Plantaginaceae	*Veronica chamaedrys*
Malpighiales	Euphorbiaceae	*Mercurialis perennis*
Malpighiales	Hypericaceae	*Hypericum perforatum*
Malpighiales	Violaceae	*Viola reichenbachiana*
Malvales	Malvaceae	*Tilia x europea*
Myrtales	Onagraceae	*Epilobium hirsutum*
Myrtales	Onagraceae	*Epilobium montanum*
Myrtales	Onagraceae	*Epilobium parviflorum*
Myrtales	Onagraceae	*Epilobium tetragonum*
Poales	Brassicaceae	*Capsella bursa-pastoris*
Poales	Cyperaceae	*Carex sylvatica*
Poales	Juncaceae	*Luzula campestris*
Poales	Poaceae	*Agrostis capillaris*
Poales	Poaceae	*Agrostis vinealis*
Poales	Poaceae	*Alopecurus pratensis*
Poales	Poaceae	*Anisantha sterilis*
Poales	Poaceae	*Anthoxanthum odoratum*
Poales	Poaceae	*Arrhenatherum elatius*
Poales	Poaceae	*Bromopsis ramosus*
Poales	Poaceae	*Bromus commutatus*
Poales	Poaceae	*Bromus hordeaceus*
Poales	Poaceae	*Dactylis glomerata*

(*Continued*)

Table 1. (Continued)

Order	*Family*	*Taxon*
Poales	Poaceae	*Deschampsia cespitosa*
Poales	Poaceae	*Elymus repens*
Poales	Poaceae	*Festuca ovina*
Poales	Poaceae	*Festuca rubra*
Poales	Poaceae	*Holcus lanatus*
Poales	Poaceae	*Lolium perenne*
Poales	Poaceae	*Melica uniflora*
Poales	Poaceae	*Phleum bertolonii*
Poales	Poaceae	*Phleum pratense*
Poales	Poaceae	*Poa annua*
Poales	Poaceae	*Poa pratensis*
Poales	Poaceae	*Poa trivialis*
Poales	Poaceae	*Vulpia bromoides*
Ranunculales	Ranunculaceae	*Clematis vitalba*
Ranunculales	Ranunculaceae	*Ranunculus acris*
Ranunculales	Ranunculaceae	*Ranunculus auricomus*
Ranunculales	Ranunculaceae	*Ranunculus bulbosus*
Ranunculales	Ranunculaceae	*Ranunculus repens*
Rosales	Rosaceae	*Agrimonia eupatoria*
Rosales	Rosaceae	*Crataegus laevigata*
Rosales	Rosaceae	*Crataegus monogyna*
Rosales	Rosaceae	*Fragaria vesca*
Rosales	Rosaceae	*Geum urbanum*
Rosales	Rosaceae	*Malus domestica*
Rosales	Rosaceae	*Potentilla reptans*
Rosales	Rosaceae	*Potentilla sterilis*
Rosales	Rosaceae	*Prunus avium*
Rosales	Rosaceae	*Prunus spinosa*
Rosales	Rosaceae	*Rosa arvensis*
Rosales	Rosaceae	*Rosa canina* agg.
Rosales	Rosaceae	*Rubus fruticosus* agg

(*Continued*)

Table 1. (Continued)

Order	*Family*	*Taxon*
Rosales	Rosaceae	*Sorbus aria*
Rosales	Ulmaceae	*Ulmus glabra*
Rosales	Ulmaceae	*Ulmus procera*
Rosales	Urticaceae	*Urtica dioica*
Sapindales	Sapindaceae	*Acer campestre*
Sapindales	Sapindaceae	*Acer pseudoplatanus*
Saxifragales	Grossulariaceae	*Ribes rubrum*
Solanales	Convolvulaceae	*Calystegia sepium*
Solanales	Convolvulaceae	*Convolvulus arvensis*
Solanales	Solanaceae	*Solanum dulcamara*
-	Boraginaceae	*Myosotis sylvatica*
Pinales	Pinaceae	*Picea abies*
Pinales	Taxaceae	*Taxus baccata*

Leaf samples from each specimen were taken for DNA extraction. High-throughput laboratory methods for 'sequencing', that is, reading, the chosen DNA barcode region from animal tissue samples (Hajibabaei et al., 2005) were adapted for plant material. Six plant DNA barcoding candidate loci – *rpoC*, *rpoB*, *rbcL*, *matK*, *trnH-psbA* and *atpF-atpH* (Chase et al., 2007; Kress & Erickson, 2007) – were isolated and amplified via PCR[11], then sequenced and deposited in GenBank (CBOL Plant Working Group, 2009).

GREAT PUCKLANDS MEADOW: 150 YEARS ON

Whether, and how, the flora of Great Pucklands meadow has changed in the 150 years since Darwin and Thorley did their survey is naturally of great interest. Have environmental changes, such as land-use change or climate change, affected a change in plant communities or phenology, that is, the timing of biological events such as flowering? Repeat surveys can reveal the biotic response to landscape and environmental change (Walther et al., 2002), and citizen scientists - young and old - may be involved in these studies; thus, a demonstration that Great Pucklands meadow has remained largely unchanged since Darwin's time would add to the justification for including not just Down House but the grounds around it in a proposed World Heritage Site.

Darwin must have made a list of the species he recorded in the meadow, for he wrote of comparing the flora of Great Pucklands with that of Ashdown Common in

Table 2. Number of seed plant species, genera, families, and orders recovered by Darwin and Thorley (1855) and the NHM team (2005-2007).

	Species	*Genera*	*Families*	*Orders*
Darwin and Thorley, 1855	142	108	-	32
NHM, 2005-2006	160	112	44	27
NHM, 2007	140	106	44	27

Sussex, noting that 'the vegetation was … considerably different in other respects, no less than nine of the 34 orders, not being found on [Great Pucklands].' (Darwin, 1857, p. 230). Alas, Darwin's species list is yet to be discovered (Randal Keynes, pers. comm.). Therefore it is only possible to compare the *number* of species, genera and orders recorded by Darwin with the number recorded during the repeat survey.

In 1855, Darwin and Thorley collected 142 seed plants belonging to 108 genera and 32 orders (Darwin, 1857, p. 230). In 2005 and 2006 the NHM team recorded 160 seed plants and one fern, the seed plants belonging to 112 genera, 44 families and 27 orders; in 2007 the NHM team re-found and collected 140 of the seed plants and the fern, the seed plants belonging to 106 genera, 44 families and 27 orders (Table 2).

Of the 160 seed plant species recorded in 2005 or 2006, 20 were not re-found (and therefore not collected) in 2007. This discrepancy may be the result of annual fluctuations in (1) the composition of the flora, (2) environmental factors that affect the timing of flowering and thus conspicuousness of some species, or (3) the identity, number of, total time spent by, and attentiveness of team members.

The number of seed plant species and genera collected by the NHM team in 2007 (140 and 106, respectively) is similar to the number Darwin reported finding in 1855 (142 and 108, respectively). At the level of order, however, the numbers diverge; the NHM team's collection spanned 27 orders while Darwin's survey spanned 32 orders. On first consideration, this might seem to suggest that the similarity between the numbers of species and genera is coincidence, and that the flora of Great Pucklands meadow changed significantly between 1855 and 2007. However, there is another potential explanation: the flora has remained relatively constant, and the NHM team and Darwin found largely the same species, but British plant taxonomy has changed in the interim, such that the orders to which those species are assigned have changed or been combined and/or renamed.

The NHM team used the second edition of Clive Stace's *New flora of the British Isles* as the primary reference for the resurvey (1997). This flora classifies British seed plants into 62 orders, while Darwin wrote that he was using a classification system with 86 orders (Darwin, 1857, p. 230). Floras he may have used as his reference include John Lindley's *Synopsis of the British flora* (1829) (Sandra Knapp, pers. comm.), one of two editions of John Stephens Henslow's *A catalogue of British plants* (1829, 1830) (Mark Spencer, pers. comm.), and the fourth or seventh edition

of William Hooker's *British flora* (1838 and 1855, respectively) (David Kohn, pers. comm.). Disappointingly, a cursory count of the orders listed in these floras fails to identify one with exactly 86; Lindley (1829) lists 103 flowering plant orders, Henslow (2nd edition, 1830) lists 98 plant orders, 91 of those being seed plant orders, Hooker (4th edition, 1838) lists 106 plant orders, 99 of those being seed plant orders, and Hooker (7th edition, 1855) lists 113 plant orders, 107 of those being seed plant orders. Thus the identity of the flora Darwin used as a reference for his survey remains elusive. Nevertheless, Stace (1997) classifies British seed plants into 24 fewer orders than the reference Darwin used, consistent with the hypothesis that changes in British plant taxonomy – rather than dramatic changes to the species assemblage of Great Pucklands meadow – underlie the difference in number of orders identified in 1855 and 2007.

Darwin went on to compare the species composition of Great Pucklands with that of 40 acres of uncultivated ground at Ashdown Common in Sussex, which was surveyed for him by 'another friend' (Darwin, 1857, p. 230). There, 106 species belonging to 82 genera and 34 orders were recorded. Darwin notes that the greater number of orders in this meadow over Great Pucklands was a result of the presence of water and marsh plants on the former. Even so, he notes 'the vegetation was … considerably different in other respects, no less than nine of the 34 orders, not being found on [Great Pucklands]' (Darwin, 1857, p. 230). A repeat of Darwin's Ashdown Common survey might add depth, breadth, and significance to the Great Pucklands re-survey.

DNA BARCODING

For DNA barcoding to be used successfully in research and its applications, two conditions must be met: the DNA barcoding region or regions must be universal, that is, present and amplifiable by PCR from the biological samples to be studied, and the DNA sequences of that region or those regions must be usable for discriminating species. Floristic and faunistic studies, that is, studies that explore the diversity of species in a geographically defined area, provide an ideal context for evaluating the universality of DNA barcoding regions because they tend to include a taxonomically broad range of species.

As a floristic study, then, the Great Pucklands meadow re-survey presented an opportunity to test the universality of a selection of candidate regions that had been proposed for use in plant DNA barcoding: *rpoC*, *rpoB*, *rbcL*, *matK*, *trnH-psbA* and *atpF-atpH* (Chase et al., 2007; Kress & Erickson, 2007). As noted earlier in this chapter, PCR amplification success rates from the Great Pucklands meadow specimens collected in 2007 have already been reported and analysed as part of a consortium-authored paper (CBOL Plant Working Group, 2009), but that paper did not allow space to report or discuss the DNA barcoding results in isolation from the larger consortium data set. Figure 3 provides an overview of PCR amplification

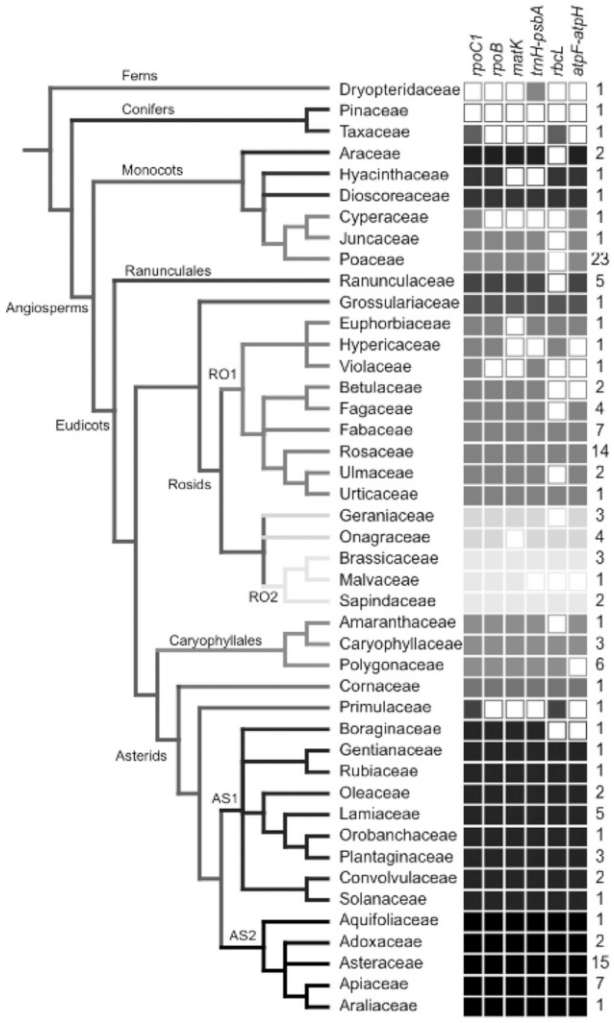

Figure 3. Phylogenetic context of included taxa and sequencing success of tested loci. The cladogram depicts the major land plant lineages (Angiosperm Phylogeny Group, 2003; Moore et al., 2007; Smith et al., 2006) modified to include only the families from which taxa were sampled for this study. The PCR amplification success of each locus is indicated, and the number of taxa sampled (n) from each family; shaded boxes indicate success in at least one taxon tested and white boxes indicate failure in all taxa tested.

success rates from the Great Pucklands meadow specimens in a phylogenetic context.

Higher PCR success rates were achieved among eudicots (one of the two major groups of flowering plants, comprising plants with seeds that bear two cotyledons) than among monocots (the other major group, comprising plants with seeds that bear one cotyledon), conifers and ferns. This result is unsurprising considering that the PCR conditions that were used had been designed for eudicots; thus, universality is highest in eudicots and decreases with phylogenetic distance. Beyond this trend, there are no other notable correlations.

In addition to providing an ideal testing opportunity for the universality of DNA barcoding regions, geographically-defined floras are also well-suited to the application of DNA barcoding for its intended purpose, that is, the identification of specimens to the species level. DNA barcoding has been shown to be more successful in floristic contexts than in the context of monographic studies, that is, studies with a geographically unrestricted but taxonomically narrow focus (Kress et al., 2009). When applied to 296 species of woody trees, shrubs, and palms found within a 50-hectare forest plot in Panama, for example, DNA barcoding was able to correctly identify 98% of specimens to the species level (Kress et. al, 2009), whereas on a global scale, just 72% of specimens can be correctly identified to the species level (CBOL Plant Working Group, 2009). The reason for this useful discrepancy is that there is often little overlap between the geographical ranges of closely related species pairs, or 'sister species', and it is the presence of sister species that is most likely to confound attempts at species discrimination through DNA barcoding in a given research context.

Thus the flora of Great Pucklands meadow holds promise for the successful application of DNA barcoding in the service of future research and/or educational endeavours at Down House. The library of DNA barcoding reference sequences generated through this project might be used in addition to or in conjunction with other technology-enabled plant identification tools such as Columbia University's prototype 'Leafsnap'[12] for the identification of plant specimens, or for the validation of identifications by 'plant-blind' students and citizen scientists (Wandersee & Schussler, 2001). This concept is being piloted as part of Tree School, a collaboration between the Natural History Museum, London, and the Cothill Educational Trust, engaging 10-15-year-old schoolchildren in building DNA barcode libraries during immersive, 5-day programmes (Hopkins & James, 2010) and BioTrails, a new collaboration of the Mount Desert Island Biological Laboratory, Acadia National Park, and the Schoodic Institute supported by an award from the National Science Foundation[13]. If implemented at Down House, such a technology-assisted citizen-science project might complement existing and proposed methods for teaching plant identification and stimulating interest and awareness (Stagg & Donkin, 2013). Findings from, and procedures developed through, such activities could in turn be applied to a wider suite of case studies addressing what Darwin called 'the awful abyss and immensity of all British Plants'.[14]

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NOTES

[1] Charles Darwin to J. D. Hooker, 15 June, 1855. Darwin Correspondence Database, http://www.darwinproject.ac.uk/entry-1700 accessed on 21 February 2010.

[2] Charles Darwin to J. D. Hooker, 5 June, 1855. Darwin Correspondence Database, http://www.darwinproject.ac.uk/entry-1693 accessed on 21 February 2010.

[3] Charles Darwin to J. D. Hooker, 15 June, 1855. Darwin Correspondence Database, http://www.darwinproject.ac.uk/entry-1700 accessed on 21 February 2010.

[4] Charles Darwin to J. S. Henslow, 27 June, 1855. Darwin Correspondence Database, http://www.darwinproject.ac.uk/entry-1705/ accessed on 21 February 2010.

[5] Charles Darwin to J. D. Hooker, 5 July, 1855. Darwin Correspondence Database, http://www.darwinproject.ac.uk/entry-1711/ accessed on 21 February 2010.

[6] Charles Darwin to J. S. Henslow, 23 (Aug–Sept, 1855?). Darwin Correspondence Database, http://www.darwinproject.ac.uk/entry-1748/ accessed on 21 February 2010.

[7] The manuscript was later presented together with extracts from Darwin's own writings on the subject of natural selection at the Linnean Society on 1 July, 1858 (Darwin and Wallace, 1858).

[8] http://www.darwinslandscape.co.uk/).

[9] Charles Darwin to J. D. Hooker, 15 June, 1855. Darwin Correspondence Database, http://www.darwinproject.ac.uk/entry-1700 accessed on 21 February 2010.

[10] Imagery © 2013 Bluesky, DigitalGlobe, Getmapping plc, Infoterra Ltd & Bluesky, Landsat, The GeoInformation Group.

[11] Polymerase Chain Reaction (PCR) is a standard molecular biology method that isolates and amplifies a region of interest for further investigation, which may include the determination of the DNA sequence of that region.

[12] (http:leafsnap.com/).

[13] Grant No. DRL-1223210.

[14] Charles Darwin to J. D. Hooker, 15 June, 1855. Darwin Correspondence Database, http://www.darwinproject.ac.uk/entry-1700 accessed on 21 February 2010.

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