THE UNIVERSITY OF HULL

Spatial and temporal variation in stomatal response to climate: a case study of modern and herbarium samples of *Salix*

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by

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1. Aims and Objectives

The purpose of this project is to investigate the interaction between atmospheric carbon dioxide (CO_2) levels and climate with the stomatal abundance of *Salix cinerea* (Grey Willow). There are three principle objectives of this research:

1. Develop a methodology for obtaining *S. cinerea* stomatal counts
    a. In terms of leaf preparation
    b. In terms of checking for effects of within-tree differences in leaf sampling on stomatal abundance data
2. Investigate the spatial variation in stomatal abundance at the present day in relation to climatic factors
3. Investigate the temporal variation of stomatal abundance over time in relation to CO_2 levels and climate factors

The first objective will involve a review of the literature of current methods used for obtaining stomatal data, coupled with testing how these methods may be adapted for application to the target species. The second objective will be achieved by quantifying the number of stomata present in modern samples collected from various locations from across Europe, which have different climates. As these samples will be collected at a similar time, any influence that CO_2 may have on the number of stomata should be mitigated and any differences present will be as a result of controlling climatic factors. The final objective will assess plant specimens collected from the same climate region, but which have been subjected to differing levels of CO_2, thus quantifying any effect that CO_2 has on the number of stomata in *S. cinerea*. This will be made possible by using samples from Hull herbarium, which contains specimens dating as far back to the early 1800s, in combination with corresponding historical climate data.

2. Background Research

2.1 Introduction

It has been estimated that for the last 30 years the global surface temperature has increased by 0.2°C every decade (Hansen *et al*, 2006). Climate change is therefore a growing concern. A severe alteration in a localised climate could result in vegetative stress and plant loss; disturbing the natural balance of an ecosystem and endangering the life cycles of directly (and indirectly) depending species. The number of stomata on plants is strongly controlled by the environment in which the leaf develops. Due to their stationary locations and long life spans, trees are an excellent indicator of their surrounding environmental conditions. Primary factors controlling the number of stomata seem to be climate and atmospheric composition. Stomatal counts can be used to reconstruct palaeo-CO_2 levels due to a strong inverse relationship between atmospheric CO_2 and the number of stomata (Woodward, 1987). Most frequently this is used for palaeoclimatic reconstructions on longer time scales (geological time), where it is not always possible to collect any direct raw data (Beerling and Woodward, 1993; Beerling and Rundgren, 2000; Retallack, 2001; Beerling and Royer, 2002; Hoof *et al*, 2004; Kouwenberg *et al*, 2005). Understanding environmental controls on stomatal density and plant phenotypical plasticity also improves prediction of possible adaptive responses to future climatic impacts.

In order to survive plants require the process of gas exchange known as photosynthesis. The cuticle (protective waxy surface layer of the leaf) however is virtually impermeable (Bird and Gray, 2003); consequently a specialist aperture is necessary to control the movement of gases in and out of the leaf during photosynthesis (Pemadasa, 1979; Nobel, 2009). Such apertures are named stomata. Stomata consist of two guard cells and a pore in between (Lawson, 2009). The guard cells have the ability to alter their turgor, which allows them to open or close (Raschke, 1979 in Sack, 1987). Alterations occur as a result of either a change in total water potential (ψ) or active changes in osmosis potential (ψ_x) (Jones, 1992). Fick's Law (Johnson and Murchie, 2011) states that the rate of this mass transfer of gas is related to the concentration gradient, as shown by the following equation:

$$J = -D\frac{\partial c}{\partial x}$$

Equation 1

Where J is the rate of mass transfer (g m^{-1} s^{-1}), D is the diffusion coefficient (m^2 s^{-1}) and $\partial c / \partial x$ is the concentration (or density) gradient of the gas (g m^{-4}). The minus indicates that diffusion occurs towards the region of lower concentration.

Stomata are predominately found in the surface of leaves, but also on some green stems (Esau, 1977). Their precise location and patterning vary between species. Generally herbaceous plants are amphistomatous, with stomata present on both the abaxial (underside) and the adaxial (topside) of the leaves (Tichà, 1982 in Lawson, 2009). Often there are slightly less on the adaxial surface (Jones, 1992). Some plants are epistomatous, with stomata on only the adaxial side. These tend to be aquatic flora, such as *Nymphaeaceae* (water lilies), which possess floating leaves. Most tree species are hypostomatous, developing stomata on the abaxial only (Morison, 2003 in Lawson, 2009).

When stomata are open (usually but not always during photosynthesis) CO_2 enters the leaf, whilst oxygen (O_2) and water vapour (H_2O) are released. In most cases when stomata are fully closed, such movement cannot occur. If stomata are partially closed then exchange takes place at a lower rate. This tends to have a greater effect on the movement of water vapour loss than the intake of CO_2 though, as the biochemical pathway for CO_2 uptake is considered longer (Fricker and Willmer, 1996). This may mean the stomata will respond quicker to a change in water availability than it would a change in CO_2 levels.

As briefly stated the number of stomata may be altered due to environmental controls (such as an abundance or lack of water). Due to developmental controls each new leaf grows with the optimal number of stomata for the conditions surrounding it. This causes variability in the number of stomata present on different plants. Further variability occurs depending on the species too. For example the angiosperm *Tilia europa* (European Lime) is thought to have a rough stomatal frequency of 370/mm^{-2} on the lower epidermis. *Pinus sylvestris* (Scots Pine) is thought to have only 120/mm^{-2} on the lower epidermis, but is thought to have the same on the upper epidermis, whereas *T. europa* has no stomata present on the upper epidermis (Mansfield and Meidner, 1968). Variation occurs between herbaceous plants too. *Allium cepa* (Onion) is reported to have 175/mm^{-2} on both upper and lower epidermal surfaces. *Vicia faba* (Broad Bean) has considerably less with 75/mm^{-2} on the lower and only 65/mm^{-2} on the upper epidermis (Mansfield and Meidner, 1968). This total may also depend on the size of the aperture, which additionally varies from species to species. For instance *Phyllitis scolopendrium* (Hart's Tongue Fern) has approximately 59/mm-2 on the lower epidermis only, with an average length of 77µm (Mansfield and Meidner, 1968); *Corylus americana* (Hazelnut) is thought to have 347 stomata mm^{-2} on the lower epidermis, but they have an average aperture length of only 37µm (Carpenter and Smith, 1975).

The number of stomata present may be quantified by using either the stomatal density (SD; the number of stomata in a specified area of the leaf surface) or the stomatal index (SI). SI, a term coined by Salisbury in 1927, is the ratio of stomata to all epidermal cells (Beerling and Chaloner, 1992). The advantage of using this method is that changes in leaf area (caused by processes such as drying and pressing a herbarium specimen) or differences in cell size between individuals alter the stomatal density but do not affect the stomatal index. Therefore the SI is a more accurate representation of how many stomata are present. Stomatal abundance can be altered by a range of environmental conditions. It may additionally be changed by a range of physical factors, such as: leaf morphology, cell size, species (Fricker and Willmer, 1996) and cuticular wax composition (Bird and Gray, 2003). It may be concluded that the SI is a more constant and reliable method of quantifying stomata, as it compensates for some of the restricting physical factors like varying cell size (and therefore more appropriate if different leaves or different samples are going to be studied).

Sometimes however it is not possible to calculate the SI, particularly when working with fossilised plant samples. Beerling *et al* (1991) make reference to the problem of surrounding epidermal cells not visibly surviving whilst the toughened structure of the stomata ensure their survival. The leaf structure of a species may also affect the method of counting stomata too. For instance, *Dryas octopetala* is frequently considered to be too hairy a species for most methods of stomata counting to be viable (Beerling and Chaloner, 1992). Jones *et al* (1995) considered acetate peel, silicone impression and scanning electron microscopy all inadequate methods of viewing stomata and epidermal cells of *Salix cinerea* because of the hairiness of the leaf, sunken stomata and complicated wax microstructure covering the surface. Despite such reservations, these methods will be tested on *S. cinerea* in the course of this project, to determine whether a method may be developed to work around such limitations.

2.2 Stomata and Climate

The presence of stomata is partially controlled by water. For instance, a shortage of water can induce the closure of stomatal pores or else a reduction in the number of stomata present. It has been noted that even in locations with adequate water levels, leaves can experience midday wilt during high incident sunlight (Smith and Young, 1980; Bell *et al*, 1997). Drought causes the release of a hormone called ABA to signal in a pathway which closes the stomata, thus reducing water loss via transpiration (Albert *et al*, 2006) because leaving the stomata open during such times would be costly for the plant (Bugs *et al*, 2011). A dysfunction are hormones like ABA can be detrimental to the plant's overall health and survival (Eissenstat *et al*, 2011). Most research in plant sciences has focused on manipulating the behaviour of the

stomata in order to isolate these genes controlling drought tolerance in *Arabidopsis* (Lascève *et al*, 1999; Azevedo *et al*, 2004; Farquhar *et al*, 2005); this is particularly useful in terms of developing drought resistant crops, such as rice, *Oryza sativa* (Dai *et al*, 2006), maize, *Zea mays* (Laporte *et al*, 2002). Chen *et al* (2007) suggests that this focus should be shifted towards the density of stomata instead, may be more efficient, as the density has proven to be of equal importance.

Another method in which water availability influences the presence of stomata is the use of crassulacean acid metabolism (CAM); a term first coined by Thomas in 1947, but was first observed during the Roman period when some house plants were noted as possessing a morning acidic taste (Black and Osmond, 2003). This is a carbon fixation pathway (Wilkins, 1993) which is a form of adaption allowing plants to survive in arid conditions (Lüttge, 1987). Flora which have adopted this technique typically have stomata which open during nocturnal hours, enabling the plant to intake CO_2 and store it as organic acids in vacuoles: stomata may then remain closed during the day, minimising water loss via evapotranspiration (Jones, 1992). CO_2 collected at night is released from the vacuoles during the following day into the Calvin cycle (the major route which carbon takes into metabolism), enabling photosynthesis to occur (Martin and Schnarrenberger, 1997). This is a process which has been observed in Cactaceae (Evans, 1932).

Other species have adapted differently with diverse thresholds. Steppe *et al*. (2007) suggest that *Quercus pubescens* (Pubescent Oak) can tolerate leaving more stomata open than *Pinus sylvestris* (Scots Pine) during dry conditions because of its ability to withdraw larger amounts of stored water from the stem and crown. However it should be noted that coniferous trees are more tolerant to soil water stress than broad-leaf trees because of their low osmotic potential, lignified (woody) guard cell walls and stomata which are sunken and suspended guard cell structure under subsidiary epidermal cells (Cai *et al*, 2002).

Temperature is also a major controlling contributor. A higher temperature would affect the stomatal abundance directly by increasing the rate of evapotranspiration, as well as indirectly via reducing the water availability. Due to climate change, a change in temperature is occurring; however this is not consistent globally. Projections made by models developed by the Intergovernmental Panel on Climate Change (IPCC) suggest that the UK will experience a 1°C increase between 2020 and 2029. During the same time period areas of Russia, north-eastern USA and northern Africa could experience an increase of 1.5-2.5°C, which may increase to a total of 6°C by 2090-2099. By this time in the UK there may be a rise of only 2.5°C (Dai *et*

al, 2001). An increase in temperature alone will probably not have too great an effect on the stomata, especially in temperate zones that will only experience minimal increases. A combination of an increased temperature and a decrease in precipitation however could have negative consequences for flora, if groundwater supplies are also low. IPCC conclude that there will probably be a general increase in the annual mean precipitation (of 15-20mm) across a belt of northern central Africa, south Asia, northern Russia, Greenland and the most northerly parts of Canada. The entirety of Australia, southern parts of North America, the Mediterranean and the very northern tip of Africa will probably experience a decrease (of 10-15mm) in annual mean precipitation (Dai *et al*, 2001). According to such predictions, countries such as Morocco, Libya and Egypt would be subjected to an increase in temperature (20°C) and a decrease in precipitation (15mm); combining this with an increasing amount of atmospheric CO_2 could potentially result in a loss of vegetation.

Other adaptations of stomata relate to the physiology of the plant, as well as particular combinations of environmental controls. For instance in conditions of high temperatures and low humidity, or excessive wind, rates of transpiration are usually high. In order to reduce water loss, the plant will close some stomata; however this causes cooling mechanisms to reduce efficiency and if prolonged may lead to the 'cooking' of the plant (TPSB, 2011). To combat this, some plants (especially in areas prone to such environmental extremes) have adapted to manipulate the microclimate around the stomata. In areas with a Mediterranean climate, most plants possess trichomes (hairs) or wax layers which reduce radiation absorbance, in addition to the stomata being sunken or well protected (Asunis *et al*, 2003).

A further example of plants adaptability due to their molecular structure is Cotton (*Gossypium hirsutum*). Plants were grown under experimental conditions in fields containing areas of high and low levels of soil salinity. The results show that high salinity caused a lower presence of epidermal cells and stomata per unit area, as well as increased the surface size of epidermal cells and greater leaf thickness (Cardenas and Gausman, 1968).

It may therefore be concluded that each species has its own threshold, up to which an individual may adapt and tolerate the conditions that it is growing in, and beyond which it simply cannot survive. It is not possible to make a general statement of how flora will respond that would be accurate for all species. Furthermore it is difficult to detangle the different aspects of climate that affect the number of stomata present, as often it is a combination of conditions that results in stomatal change.

2.3 Stomata and CO_2 Availability

Studies on the growing patterns of *Fagus* seedlings have shown that in order to cope with inflated levels of CO_2, the plants increase the water requirement to survive (Heath and Kertien, 1997; Heath, 1998). Therefore if allocation has both high temperatures and low precipitation rates, the leaves will begin to wilt to reduce the number of stomata in attempt to save water. However a plant needs a certain amount of stomata in order to photosynthesise and survive. If the plant was subjected to prolonged periods of dry conditions it may die. It should be noted that not all species respond in this way. *Pseudoroegneria spicata* (Bluebunch Wheatgrass) for example is not so greatly affected by inflated levels of CO_2 and therefore is not as sensitive to drought (Carlyle *et al*, 2009). An alternative situation would be that an area will become wetter and experience more CO_2, which may result in an increased frequency of stomata and continual flourishing of the flora. The response of stomata to climate change and an increase in toxic atmospheric gases directly relates to the specifics of how the climate will change and the species in question.

It is for this reason that stomata may be used as a proxy measure of past environmental conditions. For example, due to a strong negative relationship between increasing amounts of atmospheric CO_2 and the number of stomata (Woodward, 1987), stomatal counts may be used as a source of information on the palaeo-atmosphere and -climate. Using the known relationship between CO_2 and stomata, these 'reconstructions' of past atmospheric conditions work on the principle that a higher level of CO_2 is as a result of a higher temperature, which means a warmer climate. A limitation of this theory is that it does not consider the influence that water availability has, which is closely related to temperature and is known to affect the frequency and behaviour of stomata present. The argument for such exclusion is that it has been assumed that CO_2 is the single most important contributing factor to the number of stomata. Palaeo-climate reconstructions may be applied to a wider geological time scale, such as to the beginning of the Holocene (Beerling and Woodward, 1993; Beerling and Royer, 2002; Hoof *et al*, 2004; Kouwenberg *et al*, 2005), or even cover a period of 300 million years (Retallack, 2001). These investigations provide scope for linear regression models to be developed to predict the future response of stomata in relation to an increasing amount of atmospheric CO_2, based on such past data: models that would aid in creating a better understanding of the response of flora to such conditions.

In attempt to confirm the validity of such studies, some researchers have correlated results obtained using stomata as a proxy and direct measurements of CO_2 trapped in air bubbles within ice cores (Beerling *et al*, 1993; Barnola *et al*, 1999; Beerling and Rundgren, 2000;

Beerling *et al*, 2003; Beck, 2007). Generally the results show that leaf metabolism has not persisted at a constant state, but has fluctuated in response to shifting CO_2 concentrations (Beerling and Rundgren, 2000). It should be noted however that other environmental factors contribute to the number of stomata and there is no evidence to suggest that CO_2 is the single most important. Considering the intertwined relationship between different aspects of the surrounding climate, in addition to the effect of CO_2 on a plant, it may be suggested that these linear model predictions (even those correlated with directly measured data) are not as accurate a representation as initially anticipated. To complicate matters further CO_2 is not the only atmospheric gas that affects the number of stomata.

2.4 Stomata and Other Atmospheric Gases

Toxic gases may affect the growth rate of certain flora, which in turn affects the rate of photosynthesis and the stomata. Recent research has shown fumigation with sulphur dioxide (SO_2) resulted in a general increase in SD values in some species, but SI values were more variable depending on species (Elliott-Kingston *et al*, 2012). Such research may reduce the effectiveness of reconstructions of palaeo-atmospheric CO_2, especially during periods subjected to high levels of toxic gas in the atmosphere (Elliott-Kingston *et al*, 2012). Furthermore if this is a contributory factor, it is important to quantify the stomatal response, as levels of toxic gases (such as SO_2 and hydrogen sulphide, H_2S) may increase due to human enhanced climate change and pollution and volcanoes. It is most probable that different species have different capability thresholds to tolerate such gases (as with CO_2). The stomatal counts of *Scirpus lacustris* (Bulrush) (Bettarini *et al*, 1997) and *Agrostis canina* (Velvet Bent) showed no change in response to SO_2 and H_2S (Elliot-Kingston *et al*, 2010).

2.5 Aims of This Project

The purpose of this project is to study expression of the interaction of climate and CO_2 levels using field samples (older samples with different CO_2 'treatments' come from herbarium specimens). The species selected for this study therefore needed to have a high likelihood of appearing in the Holocene/Quaternary record (so that the findings could be applied beyond the modern period) and be well represented in the available herbarium material. The species *Salix cinerea* (Grey Willow) was selected because it grows adjacent to likely sampling contexts for Holocene/Quaternary records (wet, bog-like conditions). Furthermore there is a large collection of these species in the Hull Herbarium.

Prior to this very little research has been completed on *S. cinerea*. Jones *et al* (1995) reported that for fossilised, herbarium and modern samples of *S. cinerea* the stomatal abundance (both

SD and SI) decreased as CO_2 concentrations increased. Like so many other papers however, it does not take attempt to quantify the relationship between stomata and other contributing factors. More work has been done on *S. herbacea* (Dwarf Willow), as it has frequently been found in the fossil record, especially in the late glacial period. Modern studies show a negative relationship between the density of stomata and CO_2, and this has been used to reconstruct atmospheric levels of CO_2 throughout the Holocene (Jackson *et al*, 1983; Barnola *et al*, 1999; Beerling and Rundgren, 2000). *S. herbacea* is not the only Salicaceae to have been discovered in the fossil record though; Cevallos-Ferriz and Ramírez (2000) classified seven new taxa based on fossilised leaf remains.

The principle objectives of the project therefore are to develop the stomatal count methodology for *S. cinerea*, including preparation of the specimen. Using the most effective method of acquiring stomatal abundance, within-tree differences (if any) will be quantified and taken into consideration when carrying out the other objectives. The purpose of this investigation is to determine the influence that atmospheric CO_2 and climate have on the stomatal abundance. This will be achieved by studying the spatial variation of stomata at present day; modern samples collected from locations in Europe with different climates will provide data on how significant climatic controls are, as the samples' exposure to CO_2 should be consistent with each. The significance of the atmospheric CO_2 level will be tested using herbarium material collected from different time periods which may be assumed to have been exposed to contrasting concentrations of CO_2.

3. Materials and Methods

3.1 Stomatal density

Measures of stomatal density or stomatal index require counts of the number of stomata/epidermal cells for a selected area of the leaf surface. Due to the complexity of the species chosen, a variety of different methods have been used to obtain these counts.

Methods of obtaining stomatal counts can be divided into four categories. Stomata can be observed either directly or indirectly via a replica, and methods used can be destructive or non-destructive. Non-destructive methods leave the sample leaf intact, so that it can be resampled, and are preferable if the samples are precious (for example from a herbarium). Non-destructive direct observation methods involve using bright field or fluorescent microscopy (Karabourniotis *et al*, 2001 in Baker and Gitz, 2009), whilst destructive direct observation methods include: scanning electron microscopy (Beerling and Rundgren, 2000; Beerling *et al*, 1991; Retallack, 2001), cuticular masceration (Jones *et al,* 1995) or epidermal scraping (Eckerson, 1908 in Gitz, 2009; Travis and Weyers, 1981 in Gitz, 2009). Non-destructive indirect methods typically make an impression of the surface using a material such as acetate, nail polish, nitrocellulose lacquer, cyanoacrylate adhesive or dental putty. Destructive indirect methods involve chemical preparation of the leaf surface e.g. wax removal to prepare the specimen for taking an impression using the range of methods described above.

3.1.1 Non-destructive Direct Observation

This approach uses bright field or fluorescent microscopy (Karabourniotis *et al*, 2001 in Baker and Gitz, 2009). Whilst this method does have the advantage of not destroying samples, the main limitation is that such microscopes are expensive to purchase. It requires a microscope that not only has an external light source (or one above the mount), but also one with a high enough magnification (e.g. x 400 or greater) to view the stomata and surrounding epidermal cells. Consequently the use of microscopy for non-destructive direct observation of stomata will not be used in this investigation of *S. cinerea*.

3.1.2 Non-destructive Indirect Observation

Acetate Peel

Acetate peels are widely used to obtain impressions of surfaces of carbonate rocks (McCrone, 1963), fossilized molluscs (MacClintock and Pannella, 1968) or even human teeth (Füsun *et al*, 2005). The surface of the specimen is flooded with acetone, and then covered with a piece of

cellulose acetate which is pressed onto the leaf with even pressure. After a few minutes, the ceullose acetate sheet is peeled off the specimen and mounted on a microscope slide. The sheet surface retains an impression of the surface topography of the leaf and is translucent, so stomatal counts can be recorded using transmitted light microscopy. This technique has been successfully used to get stomatal counts from both fossil (Walton 1928, 1930; Joy et al. 1956; Beerling et al. 1995) and fresh (Beerling and Chaloner, 1992; Beerling and Woodward, 1995; Beerling and Kelly, 1997; Baker and Gitz, 2009) leaf specimens, but is not always effective with herbarium specimens. For example, Beerling *et al* (1991) tried to use acetate peels to calculate stomatal index for *Salix herbacea* leaves. The method worked well on modern leaves, but the epidermal cells on herbarium specimens were not clearly visible in the replicas, and Beerling and Chaloner (1992) conclude that the method is not suitable for species with hairy leaves. The acetate peel technique was tested in this study. Approximately 1ml liquid acetone was pipetted directly onto the leaf surface (subject to size of sample) and a sheet of acetate was rolled out on top. The acetone was left to evaporate for 15-20 minutes, and then the acetate sheet was peeled away from the leaf.

Nail Polish, Nitrocellulose Lacquer and Cyanoacrylate Adhesive

Another method that may also be unsuitable for some hairy species is the creation of a peel using clear nail polish, nitrocellulose lacquer, or cyanoacrylate adhesives. The simplicity of creating a nail polish replica makes this method accessible to non-specialists and students (Biofax, 2008; Grant and Vatnick, 2004). It has proven to work especially effectively for fresh material (Allen *et al*, 1990; Azkue *et al*, 1998; Baker and Gitz, 2009; Cuberos and Herrera, 1990; Dijkstra *et al*, 2001; Edwards *et al*, 2009; Hilu and Randall, 1984; Horanic and Gardner, 1967; Leng and Wang, 2011; Perez, 2008; Schletz, 2008; Sekiya and Yano, 2008). Applying nail polish to dried samples is less common (Miller-Rushing *et al*, 2009).

Baker and Gitz (2009) observed that spray lacquer produced similar results to that of clear nail polish, providing large replicas of *Sorghum bicolor* (sorghum) and *Glycine max* (soybean) leaves, but such peels were more difficult to prepare for *Nicotiana tabacum* (tobacco) leaves. The cyanoacrylate adhesive method is similar to skin biopsy methods (Otto *et al*, 1981). Otto *et al* (1981) recommend a coating of cyanoacrylate adhesive (commercial brands Super Glue® or Super Drop®) on the abaxial side of a specimen, which is then placed on a microscope slide with a second slide on top. A wooden clothes pin ensures pressure is administered consistently, thus producing a transparent replica of the leaf surface. An alternative form of cyanoacrylate adhesive is 151 Adhesives ™ brand wood glue (Kim, 2008) which may be less tough on fragile dried specimens.

Despite possibly being unsuitable for some hairy species (Kim, 2008), all of these methods were tested apart from 'SuperGlue' cyanoacrylate adhesive. Impressions were be made using clear nail polish and removed using clear packaging tape once the film dry (after approximately 15 minutes). Before using the nitrocellulose lacquer the spray can was shaken for 2 minutes, to ensure the mixture had not begun to separate within the bottle. The substance will then be evenly sprayed directly onto the surface of the leaf and left to dry, before being removed using clear packing tape. Some samples will be tested using two coats of the nitrocellulose lacquer. Instead of using the Super Glue® brand of cyanoacrylate adhesive, 151 Adhesives ™ wood glue will be tested. This weaker, transparent-drying wood glue will be tested on the fragile herbarium *S. cinerea* samples, in a similar way to the nail polish. The only difference between the different adhesives that were tested is the length of the drying time, which could be up 7 hours for wood glue.

Dental Material

An increasingly popular method of creating a replica uses a putty or paste to take a 'negative' or mould of the surface, then creates a cast from this mould using nail polish which can be studied using transmitted light microscopy. This process is widely used in dentistry (Altmann and Berger, 2000; Beerling and Chaloner, 1992; Black *et al*, 2002; Geisler *et al*, 2000; Green and Williams, 1988; Jackson *et al*, 2002; Jackson *et al*, 1983; Johansen and Weyers, 1985; Livingston *et al*, 2006; Perez, 2008), albeit for larger features, and multiple impressions of the same area may be made with minimal damage to the specimen.

Three brands of putty will be tested as options for obtaining data from *S. cinerea*, Hiflex DIY Putty®, Elite HD+® and Cavex Outline®. The first two are putties where two materials (the base and the catalyst) are mixed to create the moulding putty. The third is a dental impression paste. Once the putty/paste is prepared, a sample is applied and left for 5 minutes. An impression may then be made from the mould, by covering it in nail polish and leaving it for 5 minutes before removing the impression may be taken from the 'negative'. Although the first two products are essentially the same, there is a significant difference in price. Hiflex DIY Putty® is putty marketed as a DIY product, whereas Elite HD+® is putty used by professional dentists to take detailed impressions of mouths. Cavex Outline® is also used by professional dentists, although has widely been replaced by putty now.

3.1.3 Destructive Direct Observation

Scanning Electron Microscope

Scanning Electron Microscopy (SEM) is a technique suitable for fresh (Beerling and Chaloner, 1992; Beerling and Franks, 2009; Beerling and Rundgren, 2000; Bird and Gray, 2003; Björck and Rundgren, 2003; Björck *et al*, 2007; Edwards *et al*, 2009), fossil (Kerp, 1990; Leng and Wang, 2011; Retallack, 2001) and herbarium material alike (Beerling *et al*, 1991; Curtis and Lersten, 1997; Hultine and Marshall, 2001; Matamala and Peňuelas, 1990; Woodward, 1987). As biological samples are not naturally conductive, all specimens require a coat of gold, carbon or another similar metal. This may be achieved with the use of a splutter coater machine. Once prepared, the samples may be photographed in detail by a camera attached to the SEM. These are produced by placing the samples into a vacuum and firing a beam of electrons towards it. It is the rebound of these particles which creates an image, with areas of dark grey representing places of a greater conductivity (for example grooves) and those of light representing less conductive areas which reflect the electrons more easily (for example ridges). Although data obtained via this method may prove to be expensive and at the cost of the leaf, it is a technique that provides consistently high-resolution images.

Initially two 0.5cm x 0.5cm square samples were cut out of a selected range of *Salix* species leaves. These were subsequently mounted (one abaxial, one adaxial side up) onto a small circular aluminium tab mount, held in place with a carbonised sticky pad and placed inside a sputter coater machine for between 3 and 5 minutes, where a fine (2mm) layer of a gold-palladium alloy was applied. To improve the general conductivity, a small amount of colloidal silver was used in a corner of each sample. This is a paint-like paste containing tiny silver particles which is used to strengthen the conductive bond between the sample and the graphite pad that it is stuck to. After initial preparation, the coated samples could be photographed, once within the scanning electron microscope. The SEM used for this batch was an EVO 60 (Zeiss Manufacturers). The second phase of experimentation used only a single 0.5cm x 0.5cm square from each sample, as only the abaxial proved to have stomata present. For these samples a Stereoscan 360 SEM (Cambridge Instruments) was used, with an electron beam of 7.5kV. Two images taken at x500 magnification were obtained from each sample (one from the centre of the leaf and one representing the edge of the leaf sample).

Epidermal Scraping

Epidermal scraping methods use a sharp scalpel to scrape and remove the adaxial epidermis and mesophyll cells, leaving only the abaxial epidermis. Most dried leaves need to be prepared for scraping through chemical treatments to soften the tissue. The basic aim is to

destroy enough chlorophyll to ensure transparency of the leaf, whilst softening the leaf enough to remove unnecessary cells, but still be able to produce an intact layer of epidermal cells and stomata. These methods are considerably less expensive than SEM, but frequently involve harsh chemicals and require well developed fine motor skills. Many methods of preparing dried or herbarium leaves by epidermal scraping are described in the literature (e.g. Ahmad *et al*, 2011; Clark, 1960; Curtis and Lersten, 1997; Dijkstra *et al*, 1965; Hayat *et al*, 2010; Hultine and Marshall, 2001). Examples of pre-treatment include bleaching (Dilcher *et al*, 2005; Haworth *et al*, 2010) or bathing a fresh or dried leaf in 88-90% lactic acid (Ahmad *et al*, 2009; Ahmad *et al*, 2011; Clark, 1960; Dijkstra *et al*, 1965; Hayat *et al*, 2010). This technique seems to have been tested predominately on Poaceae; for this reason, none of these methods will be tried on *S. cinerea* in this project.

Some authors have developed versions of this approach suitable for both subfossil and herbarium leaves from tree species (Jones *et al,* 1995; Dilcher *et al,* 2005; Haworth *et al*, 2010). Dilcher *et al*. (2005) submerged samples in sodium hypochlorite solution for up to 12 hours before the cuticles were removed. Jones et al. (1995) found that for fossilised *S. cinerea*, overnight incubation at 60°C in a solution of hydrogen peroxide (30%) and glacial acetic acid was required, but less time is needed for herbarium or fresh examples of Cupressaceae (Haworth *et al*, 2010). As all of these methods have similar principles, only the method used by Haworth *et al* (2010) will be tested here. Samples will be placed in a test tube of 1:1 solution of 30% hydrogen peroxide (H_2O_2) and glacial acetic acid (CH_3CO_2H), at 70°C and left for an hour. Experimentation proved that this was sufficient to remove the colouration from the leaves and that any longer resulted in destruction of the cell structure. Once removed from the solution, samples were left to soak for 5 minutes in de-ionised water with a few drops of weak (10%) hydrochloric acid (HCl) and mounted on glass slides with glycerol. All chemicals will have to be neutralised in a bowl of water and sodium carbonate (Na_2CO_3) before disposal.

3.1.4 Destructive Indirect Observation

Wax Removal

Due to the development of a waxy micro-structure covering the leaf epidermal of some species a wax removal process is needed before any of the non-destructive indirect methods previously stated may be adopted. This may be achieved in a variety of different ways, including the use of collodion (Griffiths and Shepard, 2006; Haas and Rentscler, 1984; Jetter *et al*, 2000), dichloromethane (Baas *et al*, 1998; Bevilacqua *et al*, 2002; Jiang *et al*, 2009; Smart *et al*, 2008), cryo-adhesive based methods using a combination of glycerol and nitrogen (Jetter *et al*, 2000), and chloroform based removal methods (Bi *et al*, 2011; Bolck *et al*, 1994; Croteau

and Fagerson, 1971; Genet *et al*, 1997; Gołębiowski *et al*, 2008; Gołębiowski *et al*, 2012; Hietala *et al*, 1995).

The most widely used methodologies involve chloroform ($CHCl_3$), as it is a readily available chemical and has the fewest negative health implications providing a fume cupboard is utilized. The simplest methods require bathing the specimen in tepid $CHCl_3$ for various durations (20 seconds (Bolck *et al*, 1994), 45 seconds (Genet *et al*, 1997; Hietala *et al*, 1995), or 15 minutes (Baas *et al*, 1998)). Stirring may improve the effectiveness of treatment; for example, Bi *et al* (2011) stirred specimens in $CHCl_3$ continuously for 30 seconds, then transferred them to another beaker of $CHCl_3$ and stirred continuously for another 30 seconds. Heating the $CHCl_3$ may be necessary for especially waxy species. Gołębiowski *et al* (2008) successfully removed the wax residue from *Salix* leaves by submerging them for 90 seconds in $CHCl_3$ heated to 60°C. As *S. cinerea* does have a waxy cuticle, all of these $CHCl_3$ methods will be tested. To assess the success of each method both the Elite HD+® putty method (see Section 3.1.2) and the nail polish method (see Section 3.1.2) will be used on the leaf surfaces.

3.1.5 Summary of Methods

The following (Table 1) outlines a short summary reiterating the methods that were applied during this investigation.

Category of Method	Outlined in Section	Type of Method
Non-destructive Indirect Observation	3.1.2, Page 15	Acetate Peel
Non-destructive Indirect Observation	3.1.2, Page 16	Nail Polish
Non-destructive Indirect Observation	3.1.2, Page 16	Nitrocellulose Lacquer
Non-destructive Indirect Observation	3.1.2, Page 16	Cyanoacrylate Adhesive
Non-destructive Indirect Observation	3.1.2, Page 17	Putty (Hiflex DIY Putty®)
Non-destructive Indirect Observation	3.1.2, Page 17	Dental Material (Elite HD+®)
Non-destructive Indirect Observation	3.1.2, Page 17	Dental Material (Cavex Outline®)
Destructive Direct Observation	3.1.3, Page 18	Scanning Electron Microscope
Destructive Direct Observation	3.1.3, Page 18	Epidermal Scraping
Destructive Indirect Observation	3.1.4, Page 19	Wax Removal

Table 1: A summary of methods that were tested on leaf samples, in order to obtain SD counts.

4. Leaves Sampled

4.1 Origin of Leaf Samples

The countries from which samples were collected from include: Britain, Ireland, France, Germany and Sweden. Each individual tree that twig have been obtained from has been allocated a letter which has been used to highlight the site location in Figures 2-7. These labels have been colour coordinated in accordance to the species of the sample, which may be found in Figures 8 and 9. This investigation is part of a larger umbrella project, which has connections with institutes in the previously mentioned countries and was able to provide travel funding to these locations. The contrasting geographical distributions provided scope for analysing the stomatal abundance in different climate zones (addressing the second principle aim, see Section 1). Although all the visited countries have temperate climates, on a more local scale the sites have slight differences. For instance, the Irish specimens have been exposed to a wet oceanic weather regime. The German specimen grew in a generally drier, continental environment.

All *S. cinerea* samples were collected between late spring (April) and early autumn (September). If they were collected any earlier in the year, the leaves may have not reached full maturity and would have skewed the results. Equally any later during the year, the leaves would be dying back and falling in preparation for the winter.

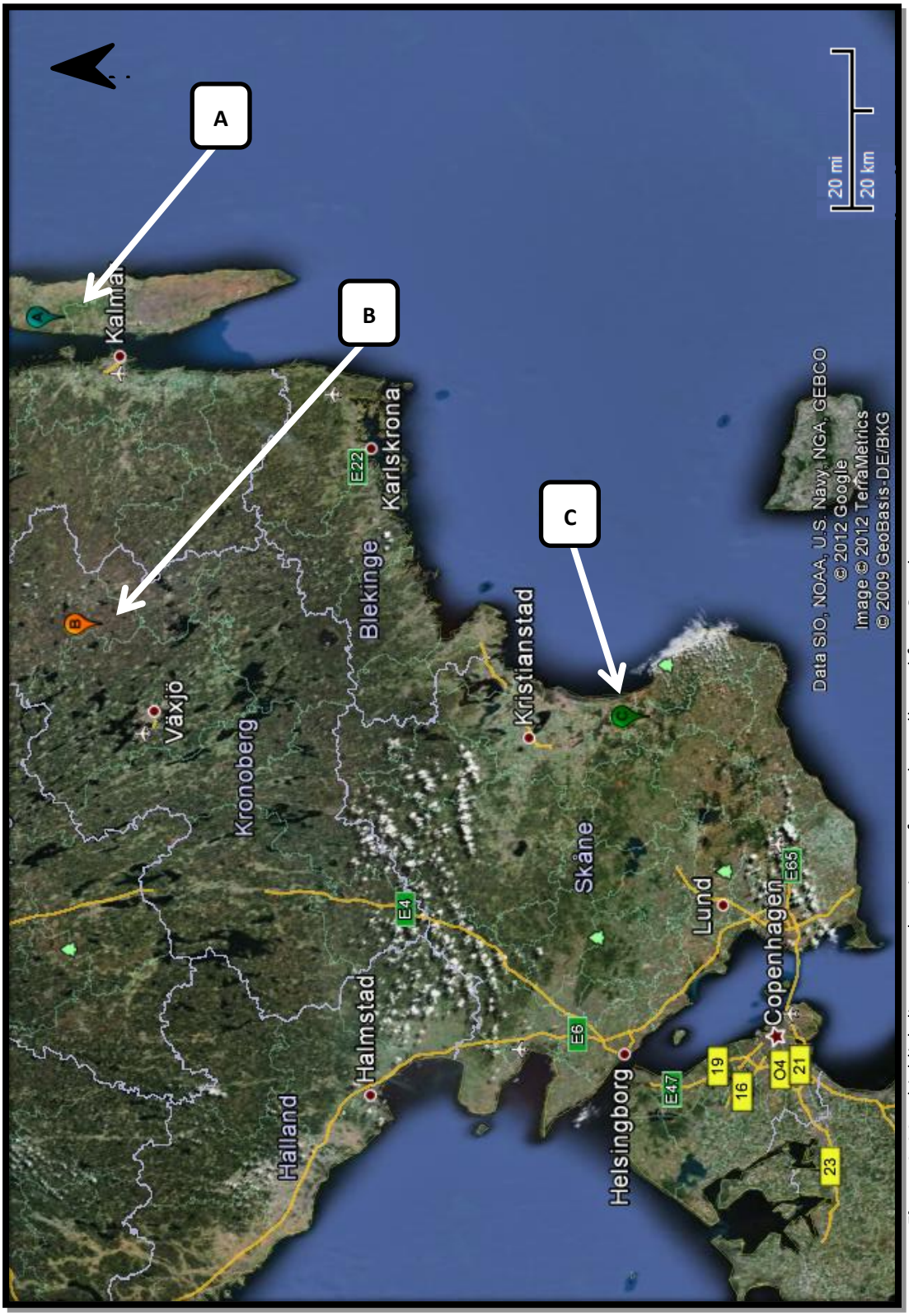


Figure 1: Map highlightling exact locations of samples collected from Sweden.

Figure 2: Map highlighting exact location of the sample collected from Germany.

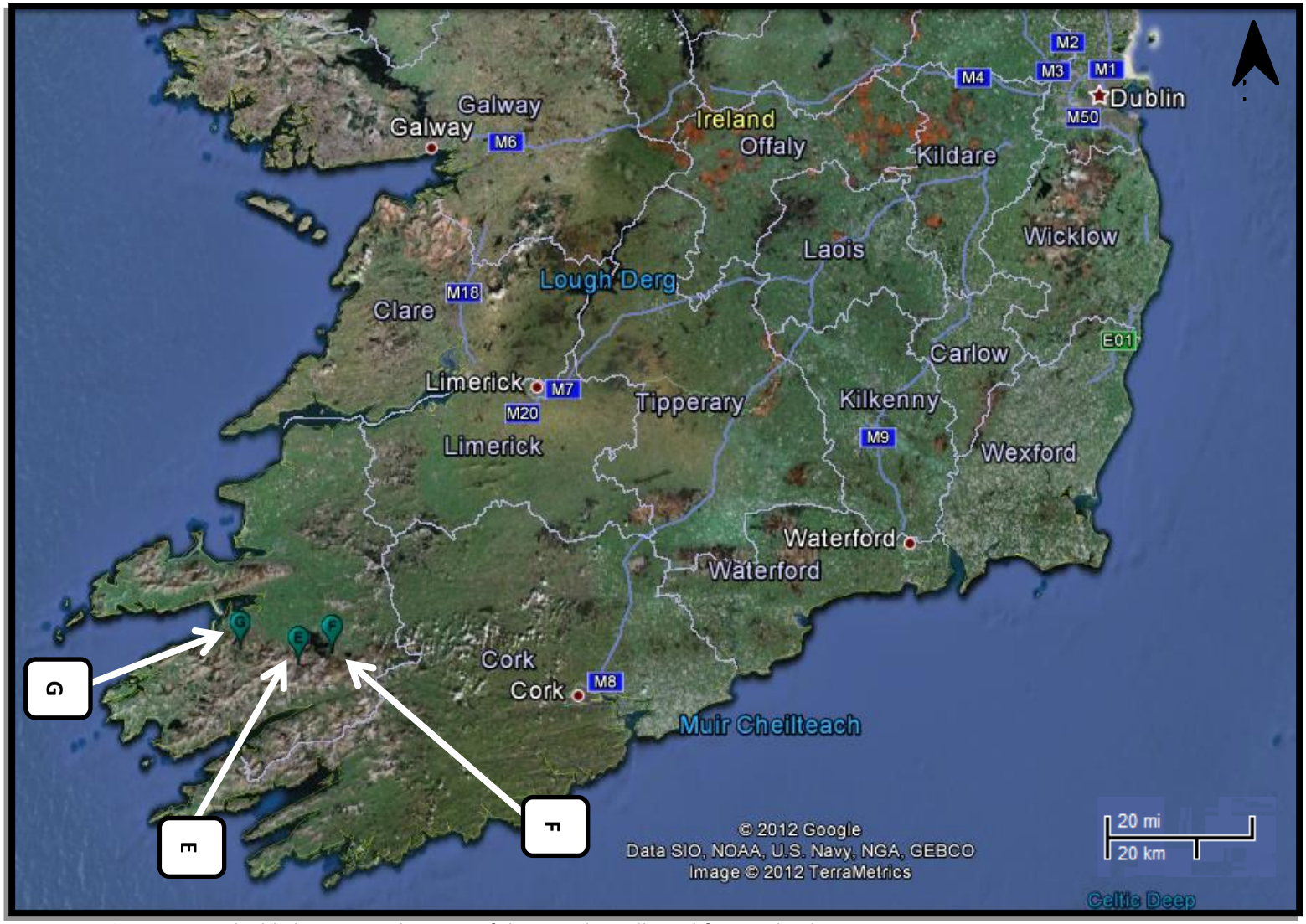


Figure 3: Map highlighting exact location of the samples collected from Ireland.

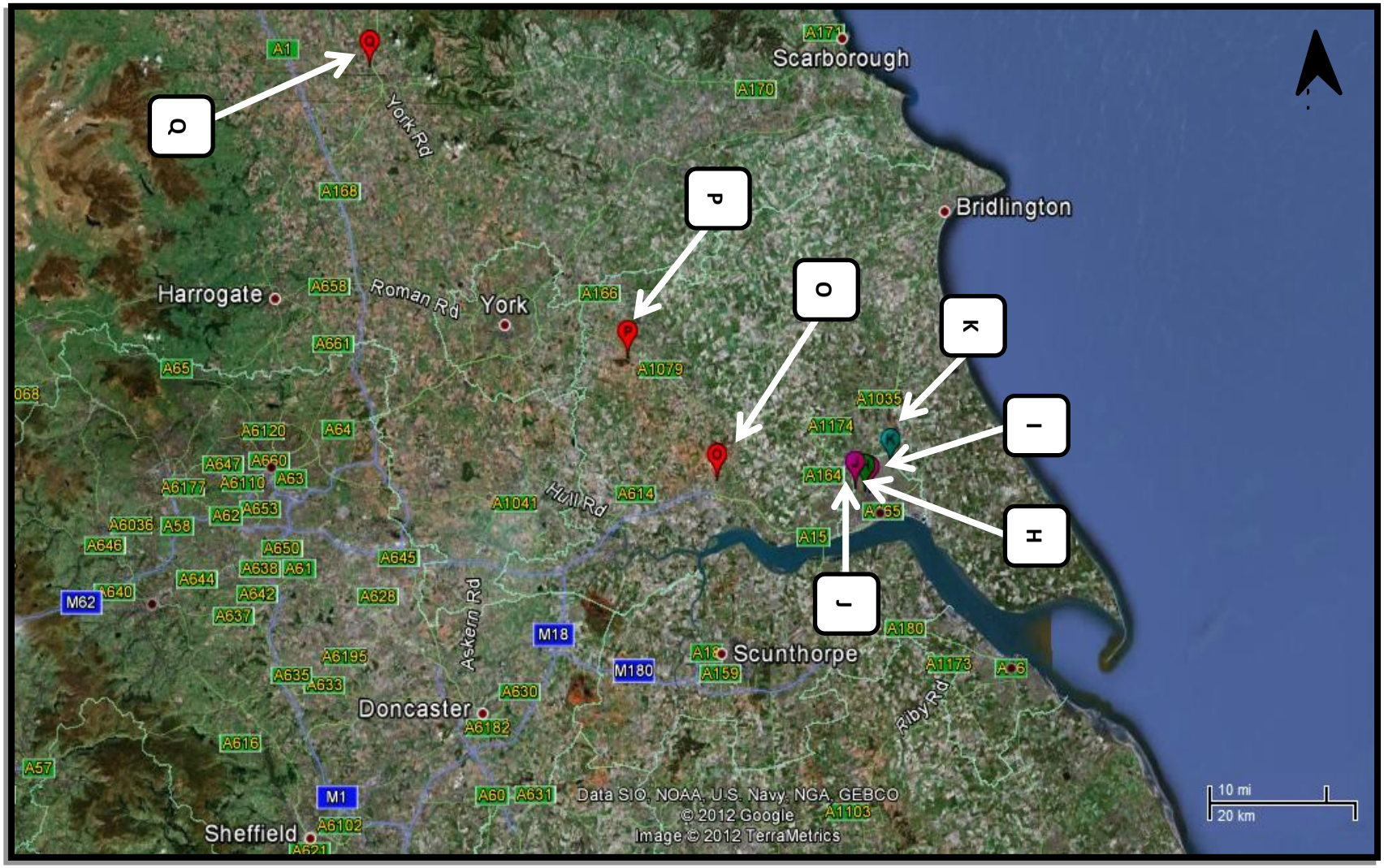


Figure 4: Map highlighting exact location of the samples collected from North-Eastern England.

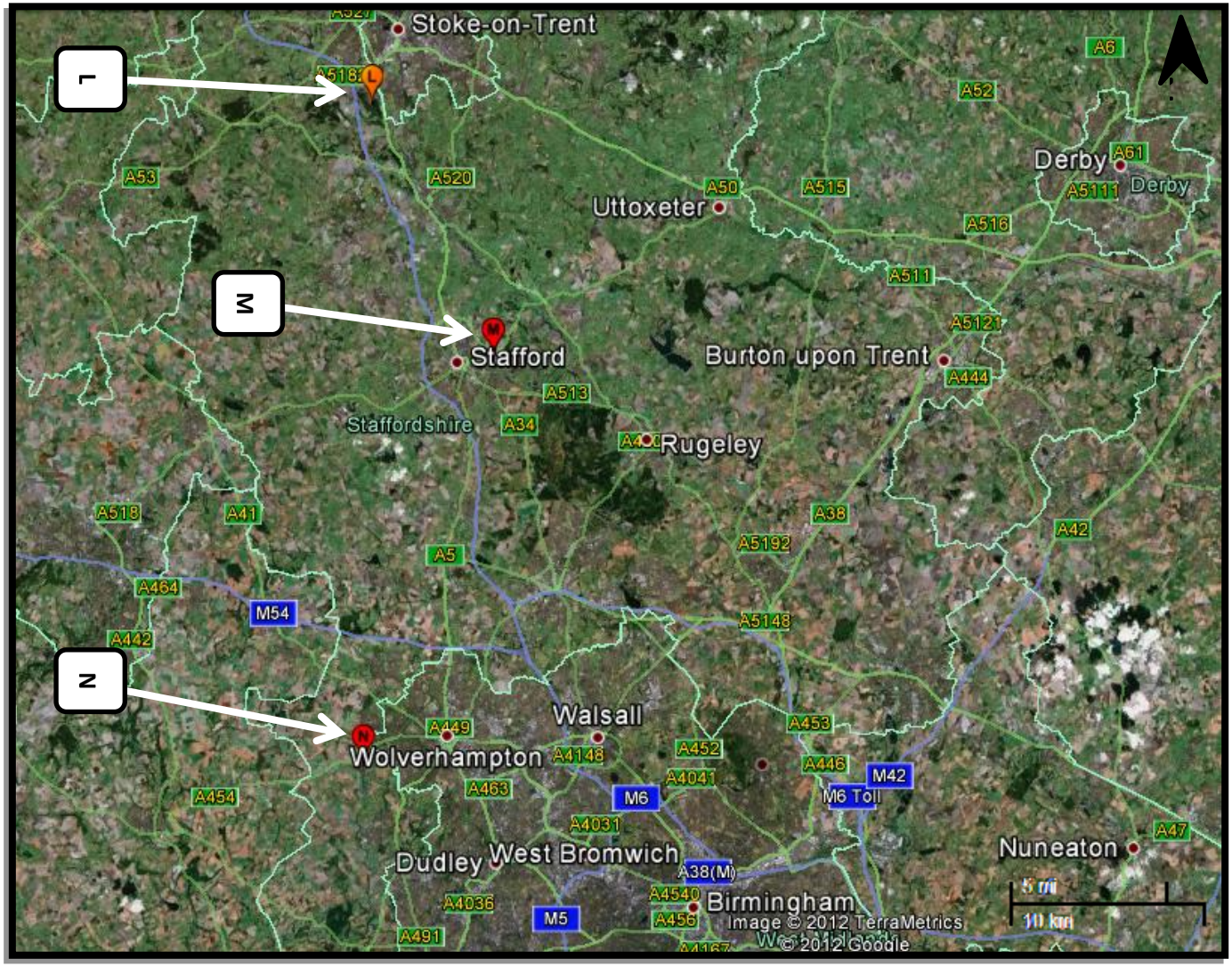
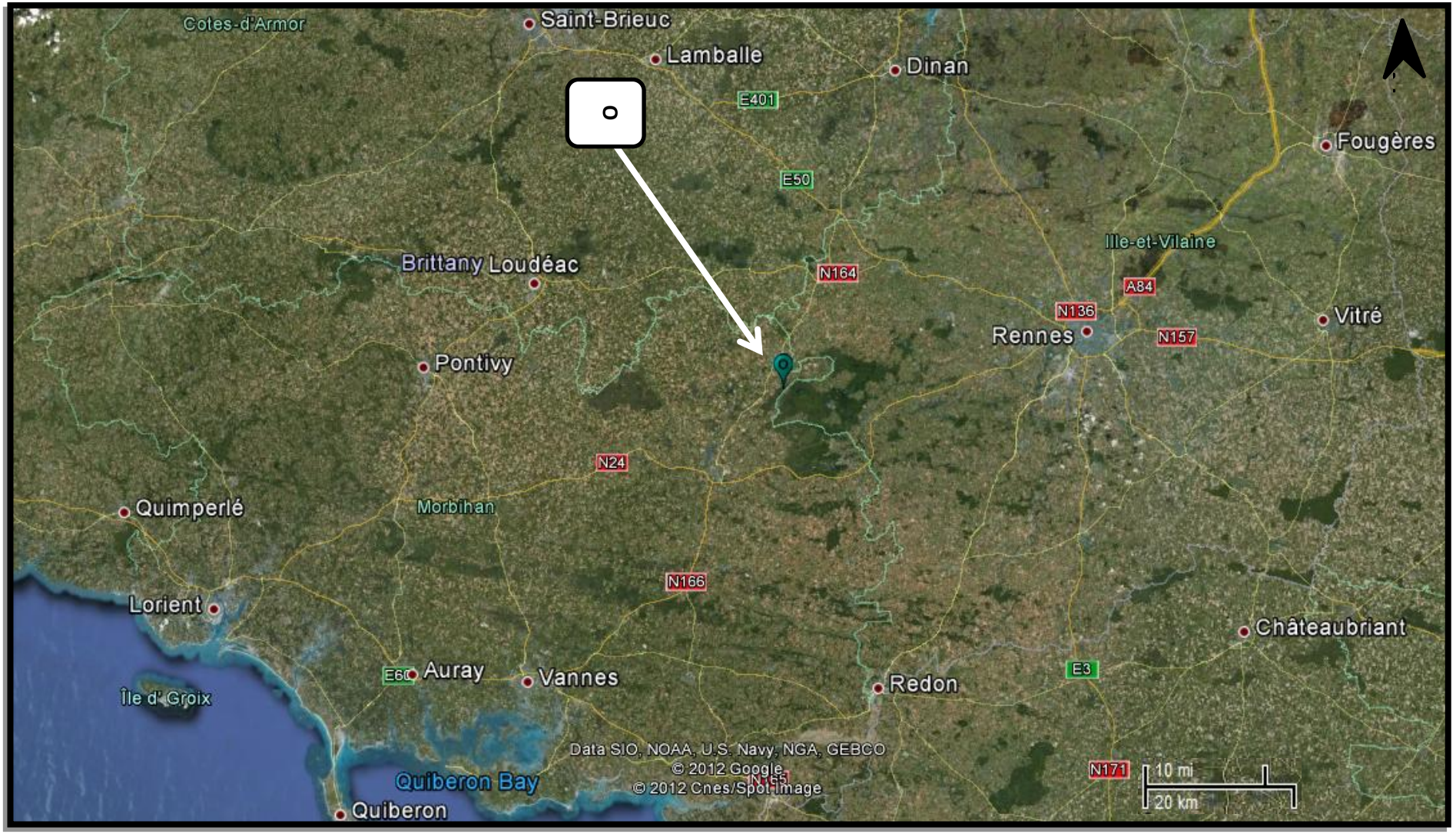


Figure 5: Map highlighting exact location of the samples collected from Central England.


Cotes-d'Armor
Saint-Brieuc
Lamballe
Dinan
E401
Fougères
E50
Ille-et-Vilaine
Brittany Loudéac
N164
A84
N136
Vitré
Rennes
N157
Pontivy
N24
Quimperlé
Morbihan
N166
Lorient
Châteaubriant
E60
Auray
Vannes
Redon
E3
Île d' Groix
Data SIO, NOAA, U.S. Navy, NGA, GEBCO
© 2012 Google
© 2012 Cnes/Spot Image
N165
N171
Quiberon Bay
Quiberon
10 mi
20 km

Modern Samples

Map Label	Date Collected	Sample Number	GPS Co-ordinates	Species	Site Name	Site Description
A	05/07/2011	B027	N56° 45' 17.7", E016° 36' 52.9"	*Salix cinerea*	Mittlandsk-öggen, Sweden	East side of track, growing from abandoned wall parallel to a ditch
B	11/07/2011	B112	N56° 55' 41.1", E015° 15' 11.2"	*S.* cf. *caprea*	Storesjö, Sweden	Side of track near car park
B*	11/07/2011	B113	N56° 55' 41.1", E015° 15' 11.2"	*S.* cf. *caprea*	Storesjö, Sweden	Side of track near car park, next to previous sample
C	16/07/2011	B172 (CCC)	N55° 43' 54.8", E014° 04' 37.2"	*Betula pendula*	Brösarps Backar, Sweden	Edge of mixed woodland, along a small track
C*	16/07/2011	B167 (CCC)	N55° 43' 54.8", E014° 04' 37.2"	*Quercus robur*	Brösarps Backar, Sweden	South edge of a car park, bordering mixed woodland
D	11/08/2011	B217	N48° 2' 36.1", E8° 31' 32.9"	*S. cinerea*	Schwenningen Moos, Germany	Side of track leading towards Schwenningen Moos
E	10/09/2011	SS2 (B309)	N51° 58' 46.6", W009° 38' 03.6"	*S. cinerea*	Lord Brandon's Cottage, Ireland	River bank near Lord Brandon's Cottage
F	11/09/2011	B310	N52° 00' 27.3", W009° 28' 49.5"	*S. cinerea*	Mangerton, Ireland	Part of mountain track, near stream
G	13/09/2011	B347	N52° 1' 02.9", W009° 51' 18.4"	*S. cinerea*	Muckross Peninsula, Ireland	Margins of wet, flat low-lying area
H	14/10/2011	Betula	N53° 46' 11.3", W000° 21' 53.3"	*B. pendula*	Salmon Grove, Yorkshire	Seasonally flooded semi-open woodland, near car park of the University of Hull

Table 2: A description of all of the modern samples experimented with, including the exact locations and date of collection.

Modern Samples continued

Map Label	Date Collected	Sample Number	GPS Co-ordinates	Species	Site Name	Site Description
I	03/11/2011	Salix sp. 1	N53° 46' 5.9", W000° 21' 21.3"	*S. x sepulcralis*	Cottingham Road, Yorkshire	Small grassland area behind the church, near the car park
J	31/01/2012	Salix sp. 2	N53° 46' 26.0", W000° 23' 3.0"	*S. x sepulcralis*	Owston Park, Yorkshire	Residential garden, bordering car park behind house
K	29/07/2012	SS5	N53° 47' 54.7", W000° 18' 46.8"	*S. cinerea*	Noddle Hill, Yorkshire	Bordering footpath surrounding fishing lake
L	27/06/2012	SS6	N52° 57' 28.0", W002° 12' 16.7"	*S.* x *reichardtii*	Trentham, Stafforshire	Edge of a damp wood, bordering a landscaped lake, near River Trent
O	28/06/2012	B445	N48° 02' 22.6" W002° 16' 02.1"	*S. cinerea*	La Ferme du Parc Jacques, France	A strip of roadside scrub, bordering a managed conifer woodland and an area of open gorse dominated heath
*Note: Sample letters have been duplicated due to the GPS of the samples being the same, so one label has been used for both samples.						

Table 2 continued: A description of all of the modern samples experimented with, including the exact locations and date of collection.

Herbarium Samples

Map Label	Sample Number	Catalogue Number	Species	Date	Site Name	Site Description
M	SS1	17167a (Box 5)	*Salix cinerea*	21/08/1885	Kingston Pool, Stafford	A pool surrounded by wet woodland, but has recently become urbanised
M*	SS3	17167a (Box 5)	*S. cinerea*	20/04/1886	Kingston Pool, Stafford	A pool surrounded by wet woodland, but has recently become urbanised
N*	SS4	17165a (Box 5)	*S. cinerea*	22/08/1884	Pool Hall Pool, Staffordshire	A series of managed pools, surrounded by wet scrubland, but is now a fishery
N*	L24	17160 (Box 5)	*S. cinerea*	07/09/1883	Pool Hall Pool, Staffordshire	A series of managed pools, surrounded by wet scrubland, but is now a fishery
O	L20	6860 (Folder 343, Sleeve 12)	*S. cinerea*	?/06/1955	North Cave, Yorkshire	An open area of scrubland and ponds, bordering a small village
P	L21	6861 (Folder 343, Sleeve 12)	*S. cinerea*	13/04/1931	Barmby Moor, Yorkshire	A small area of mixed woodland with a wet area in the centre
Q	L22	17151 (Box 5)	*S. cinerea*	?/?/1854	South Kilvington, Yorkshire	River bank of River Cam near its source, not far from the North York Moors
L**	L23	17158 (Box 5)	*S. cinerea*	22/04/1873	Trentham, Staffordshire	Edge of a damp wood, bordering a landscaped lake, near River Trent

*Note: Only one label has been used to mark this site, as the same tree was probably resampled, although this cannot be confirmed.
**This sample shares the same label as the modern Trentham sample, as the locations are within very close proximity.

Table 3: A description of all of the herbarium samples which were experimented with, including their collection date and catalogue number.

No stomatal abundance data were obtained from *S. cinerea* using non-destructive indirect methods (see Section 4.2). To determine whether this was due to the choice of species or an incorrect application of method, *Betula pendula* (Silver birch) and *Quercus robur* (Pedunculate oak) were also tested on. *S.* x *sepulcralis* (Weeping willow), a hybrid between *S. babylonica* (Chinese weeping willow) and *S. alba* (White willow), was also tested. Table 2 and 3 also make reference to samples of *S. caprea* (Goat willow) and *S.* x *reichardtii* (a Sallow hybrid); this is due to a conscious decision to make no distinction between different varieties of 'Pussy Willows' because of the genetic similarities and difficulties in identification.

Generally *Salix* taxonomy is notoriously difficult (Bebbington and Sinker, 1979). British populations of *S. cinerea* may be sub-divided into two relatively distinct subspecies: *Salix cinerea* L. ssp. *cinerea* (Grey Sallow) and *Salix cinerea* L. ssp. *oleifolia* Macreight (Rusty Sallow) (Meikle, 1984). For this investigation whilst each subspecies may be identified, it has not been acknowledged because they are so genetically similar. A large proportion of the samples collected from the Continent were subspecies *cinerea*, whereas the British examples were more often subspecies *oleifolia*, as it is more common in the UK (Stace, 2010). The problems arise with the subspecies *oleifolia*. This is because it often hybridises with *S. caprea* (Goat Willow). Not only does it hybridise, it can back-cross freely with either parent and has a tendency to replace *S. caprea* (Dines *et al*, 2002). Often a specimen may appear to be *S. caprea*, but it is extremely difficult to determine whether it is a hybrid (Bean, 1980).

Even the microstructure of these species is similar. Figure 11 (in Appendix 1) shows a SEM image of L7 (the abaxial side of a *S. caprea x S. cinerea* = *S.* x *reichardtii* hybrid collected from Trentham, Staffordshire) and Figure 19 (in Appendix 1) is a SEM image of L23 (the abaxial side of a *S. cinerea* ssp. *oleifolia* leaf also obtained from Trentham, Staffordshire). These images illustrate how similar the wax structures are to each other, the only difference is that *S.* x *reichardtii* has slightly finer 'conicoids' than the pure *S. oleifolia*. Where this is due to *S. caprea* having a different wax structure, or just because this sample has more hair follicles is unclear. In retrospect some of the *S. oleifolia* samples that have been collected may in fact be to some extent a hybridisation. Results of these have however not been discarded because the genetics remain so similar. This does raise questions about the organisation of these Salicaceae. Should these be reclassified as an aggregate? Often both subspecies of *S. cinerea* along with *S. caprea* are called 'Pussy Willow' due to their silky grey buds which resemble cats' paws (Sterry, 2007). If they are so closely associated, then should not be regrouped? Although the two 'races' of *S. cinerea* may be relatively easy to distinguish from each other, in West Britain the distinction becomes far less obvious (Meikle, 1984). Furthermore the subspecies *cinerea* (on the

Continent) may often be confused with *S. aurita* L. x *S. cinerea* L. ssp. *oleifolia* Macreight. So problems with identification of a 'pure' species may not necessarily be mitigated by only collecting the *S. cinerea* ssp. *cinerea*.

In some cases the habitat may be used to help determine the species. *S. caprea* is usually found in hedgerows, by woodland margins, rocky lakeshores, on well drained ground, in woods which have been felled and well disturbed, although it can occur on wetter ground too (MobileReference, 2009). *S. cinerea* ssp. *oleifolia* prefers the edges of bogs and marshes, or moist woodland margins, hedgerows, or stream sides (Meikle, 1984). Although Mitchell (1992) states that *S. cinerea* has a similar distribution to *S. caprea* both in Britain and in Europe. So the habitat may provide little aid in identification. An example of this is at Trentham. This is an old country estate, which the River Trent flowed directly through. The river was managed and diverted by Reverend George Plaxton in 1695, creating a pair of canals with a gravelled walkway between them. Between 1759 and 1780 Lancelot 'Capability' Brown redeveloped the grounds and enlarged the pre-existing lake. The area surrounding the lake consists of ancient woodland remnants, which is now heavily managed and has a reasonably sized population of wild Black Fallow Deer (*Dama dama*) (Trentham Leisure Ltd., 2012). The location where the *S.* c.f. x *reichardtii* samples were collected from was near the edge of the woodland margin, however it was difficult to assess how moist the soil usually is because the year of collection (2012) was a dry year, until the summer months which were then the wettest for a hundred years (BBC, 2012).

4.2 Effects of Sampling Strategy on Measurements of Stomatal Density

This study aims to investigate the effects of climate on stomata density for *S. cinerea*, and determine whether variations in stomatal density over the last 150 years can be explained better by including climate data as well as CO_2 levels in a regression model. This will be achieved in four stages.

Step 1 is to determine the effects of sampling strategy on stomatal density using samples from one location. This stage is particularly important as it will determine the sampling methods which will be adopted for the other samples. As the sampling strategy may affect the results, the specimens must be consistently sampled. This can be problematic using stomatal counts as a measure of stomatal density, since it is thought that SD estimates made from dried leaves (such as those from a herbarium) potentially overestimate the density compared to fresh leaves (Hultine and Marshall, 2001) because leaves may shrink on drying, leading to more stomata being counted in the same area after drying. It is not clear whether dried leaves shrink

at a consistent manner if pressed or air-dried and for different lengths of drying time; if all leaves shrink equally on drying, then dried samples can be compared, but if not then it may be worth considering rehydrating all of the samples before counting. However, this step assumes that the leaves would all expand to their original size and shape, regardless of how they were dried or length of storage since drying. These problems could to a certain extent be avoided if the SI was counted instead, because the SI is concerned with the ratio of stomata to other epidermal cells in an area. In theory if the area is the same, then the results should be consistent. In this investigation however, this is not possible. The hypotheses that will be tested are:

- $\mathbf{H_1}$ = There is a statistically significant difference between the frequency of stomata present on the edge of a leaf and in the middle of a leaf.
- $\mathbf{H_{01}}$ = There is no statistically significant difference between the frequency of stomata present on the edge of a leaf and in the middle of a leaf.

- $\mathbf{H_2}$ = There is a statistically significant difference between the frequency of stomata present on different leaves on the same twig.
- $\mathbf{H_{02}}$ = There is no statistically significant difference between the frequency of stomata present on different leaves on the same twig.

- $\mathbf{H_3}$ = There is a statistically significant difference between the frequency of stomata present on leaves which have been pressed and leaves which have not been pressed.
- $\mathbf{H_{03}}$ = There is no statistically significant difference between the frequency of stomata present on leaves which have been pressed and leaves which have not been pressed.

- $\mathbf{H_4}$ = There is a statistically significant difference between the frequency of stomata present on leaves of the same position on the twig on different twigs from the same larger branch.
- $\mathbf{H_{04}}$ = There is no statistically significant difference between the frequency of stomata present on leaves of the same position on the twig on different twig from the same larger branch.

- $\mathbf{H_5}$ = There is a statistically significant difference between the frequency of stomata present on leaves in the same position on a twig from different samples.
- $\mathbf{H_{05}}$ = There is no statistically significant difference between the frequency of stomata present on leaves in the same position on a twig from different samples.

Some of these hypotheses will be revisited during later stages. For each hypothesis, stomatal density values will be determined for leaves collected from positions identical in all factors except the one being explored (e.g. to test H_2 impressions will be taken from the same location on leaves collected from the same position on the twig which have been either pressed or dried without pressing). The mean values of stomatal count per unit area and stomatal indices were compared statistically using the Student's *t*-test (where to conditions are to be compared) or one way Analysis of Variance (ANOVA) (where three or more conditions are compared) (Field, 2011). This will be achieved using the IMB's statistical program SPSS. Table 4 states what statistical test is appropriate for each hypothesis that will be tested.

Hypothesis	Statistical Analysis Test
H_1	Student's T-Test
H_2	One Way ANOVA
H_3	Student's T-Test
H_4	One Way ANOVA
H_5	Student's T-Test

Table 4: A list of statistical tests used to test each hypothesis.

4.3 Effects of Climate on Stomatal Density in Field Specimens

Step 2 is to determine whether climate affects stomatal density for modern samples using climate data and stomatal density data. Sample collection locations are discussed in Section 3.2. In order to do this, climate data for the general localities has been collected from a range of sources, see Table 5.

Location Sampled	Distance from *Salix*	Year Data Collected	Data Available	Source of Data
Munich Riem, Germany	226.62km East from Site D	March, April and May 2011	Daily data: mean temperature (T) (°C), maximum temperature (TM), minimum temperature (Tm), mean humidity (H), precipitation amount (PP) (mm), mean visibility (VV) (km), mean wind speed (V) (km/h), maximum sustained wind speed (VM), maximum wind gust (VG), indicator for occurrence of rain or drizzle (RA), indicator for occurrence of thunder (TS), indicator for occurrence of fog (FG) and monthly mean data	Tutiempo. http://www.tutiempo.net/en/Climate/Munich_Riem/03-2011/108660.htm (Accessed 21st August 2012).

Table 5: Sources used to obtain climate data from near the sampled sites.

Location Sampled	Distance from *Salix*	Year Data Collected	Data Available	Source of Data
Munich, Germany	226.62km East from Site D	March, April and May 2011	Monthly Data: Hours of Daylight (L)	Time and Date http://www.timeanddate.com/worldclock/astronomy.html?n=168&month=3&year=2012&obj=sun&afl=-11&day=1 (Accessed 26th September 2012).
Valentia Observator, Republic of Ireland	51.02km West South West from Site E, 61.15km West South West from Site F, 36.44km South West from Site G	March, April and May 2011	Daily data: T, TM, Tm, mean sea level pressure (SLP) (hPa), H, PP, VV, V, VM, VG, RA and monthly mean data	Tutiempo. http://www.tutiempo.net/en/Climate/VALENTIA_OBSERVATOR/03-2011/39530.htm (Accessed 21st August 2012).
Limerick, Republic of Ireland	101.13km North North East from Site E, 90.85km North North East from Site F, 108.68km North East from Site G	March, April and May 2011	Monthly Data: L	Time and Date http://www.timeanddate.com/worldclock/astronomy.html?n=1964&month=3&year=2011&obj=sun&afl=-11&day=1 (Accessed 26th September 2012).
Växjö, Sweden	110.40km West from Site A, 28.61km South West from Site B, 131.31km North North East from Site C	March, April and May 2011	Daily data: T, TM, Tm, H, PP, VV, V, VM, VG, RA and monthly mean data	Tutiempo. http://www.tutiempo.net/en/Climate/Vaxjo/07-2011/26410.htm (Accessed 21st August 2012).

Table 5 continued: Sources used to obtain climate data from near the sampled sites.

Location Sampled	Distance from *Salix*	Year Data Collected	Data Available	Source of Data
Växjö, Sweden	110.40km West from Site A, 28.61km South West from Site B, 131.31km North North East from Site C	March, April and May 2011	Monthly Data: L	Time and Date http://www.timeanddate.com/worldclock/astronomy.html?n=1391&month=3&year=2011&obj=sun&afl=-11&day=1 (Accessed 26th September 2012).
Humberside, England	23.47km South from Site H, 23.25km South from Site I, 24.04km South South East from Site J, 26.48km South South West from Site K	March, April and May 2012	Daily data: T, TM, Tm, H, PP, VV, V, VM, VG, RA, TS, FG and monthly mean data	Tutiempo. http://www.tutiempo.net/en/Climate/Humberside/03-2012/33735.htm (Accessed 21st August 2012).
Kingston-upon-Hull, Humberside	6.13km South South West from Site K	March, April and May 2012	Monthly Data: L	http://www.timeanddate.com/worldclock/astronomy.html?n=1319&month=3&year=2012&obj=sun&afl=-11&day=1 (Accessed 26th September 2012).
Rennes, France	44.78km West South West from Site O	March, April and May 2012	Daily data: T, TM, Tm, SLP, H, PP, VV, V, VM, VG, RA, FG and monthly mean data	Tutiempo. http://www.tutiempo.net/en/Climate/Rennes/03-2012/71300.htm (Accessed 2nd September 2012).

Table 5 continued: Sources used to obtain climate data from near the sampled sites.

Location Sampled	Distance from *Salix*	Year Data Collected	Data Available	Source of Data
Rennes, France	44.78km West South West from Site O	March, April and May 2012	Monthly Data: L	Time and Date http://www.timeanddate.com/worldclock/astronomy.html?n=1264&month=3&year=2012&obj=sun&afl=-11&day=1 (Accessed 26th September 2012).
Thornecliffe, near Leek, England	23.24km North East from Site L, 35.13km North North East from Site M, 62.71km North East from Site N	March, April and May 2012	Daily data: T, TM, Tm, SLP, H, PP, VV, V, VM, VG, RA, FG and monthly mean data	Tutiempo. http://www.tutiempo.net/en/Climate/LEEK_THORNCLIFFE/03-2012/33300.htm (Accessed 27th August 2012).
Central England	Birmingham (the centre of the CET) is 56.73km South East from Site L, 38.49km South South East from Site M, 23.45km East South East from Site N, 166.45km South West from Site O, 174.50km West South West from Site P, 199.34km South South West from Site Q	January 1659-December 2011	Monthly data: T and daily data: T	MET Office Hadley Central England Temperature Data. http://www.metoffice.gov.uk/hadobs/hadcet/data/download.html (Accessed 21st August 2012).
Oxford, Oxfordshire	151.98km South East from Site L, 132.57km South East from Site M, 113.87km South East from Site N, 283.58km South from Site Q	January 1853-December 2011	Monthly Data: TM, Tm and PP	MET Office http://www.metoffice.gov.uk/climate/uk/stationdata/oxforddata.txt (Accessed 12th September 2012).

Table 5 continued: Sources used to obtain climate data from near the sampled sites.

Location Sampled	Distance from *Salix*	Year Data Collected	Data Available	Source of Data
Sheffield, Yorkshire	70.92km West South West from Site O and 75.12km South West from Site P	January 1883-December 2011	Monthly Data: TM, Tm and PP	MET Office http://www.metoffice.gov.uk/climate/uk/stationdata/sheffielddata.txt (Accessed 12th September 2012).
Stoke, Stoke-on-Trent	5.16km North North West from Site L	March, April and May 2012	Monthly Data: L	Time and Date http://www.timeanddate.com/worldclock/astronomy.html?month=3&year=2012&obj=sun&afl=-11&day=1&n=1321 (Accessed 26th September 2012).

Table 5 continued: Sources used to obtain climate data from near the sampled sites.

It should be noted that the MET Office CET only provides past temperature data of Central England. As precipitation data was also required for analysis, another source of information had to be found. The closest location that had records that went back far enough to relate to the herbarium samples was Oxford. Despite being over 100km from the samples collected from Staffordshire, the weather station is far enough inland to have a relatively similar climate and lies within the triangular perimeter of the CET. Additional data was obtained from a weather station in Sheffield, as this is closer to the Yorkshire sites, and would have been ideal for the Staffordshire samples too, but unfortunately this data set is not quite old enough. It is because of this that climate data for Site Q will have to be analysed in collaboration with the Oxford data. The monthly total of rainfall and mean temperature for the spring months prior to collection will be analysed in relation to the stomatal density. The recorded hours of daylight (for modern samples) will be used as a proxy for a light availability. Additionally the growing season total (the length of time which the specimen will continue to grow in) will also be calculated and compared to the SD results.

A simple linear regression model will be developed to determine whether variations in stomatal density can be explained in terms of climate factors. A linear regression model takes the form:

$$Y_i = (\beta_0 + \beta_1 X_i) + \varepsilon_i$$

Equation 1

Where Y is the outcome variable (a measure of stomatal density); X is the predictor variable (climate factor); β_1 is the regression coefficient associated with the predictor; β_0 is the value of the outcome when the predictor is zero and ε_i is error (Field, 2011). This model can be extended to incorporate multiple climatic factors, as follows:

$$Y_i = (\beta_0 + \beta_1 X_{1i} + \beta_2 X_{2i} + \cdots + \beta_n X_{ni}) + \varepsilon_i$$

Equation 2

Whereby: Y is the outcome (stomatal density); X_1, X_2 etc. are separate predictors (e.g. total precipitation, mean monthly temperature); β_{ni} are regression coefficients associated with each predictor variable; β_0 is the value of the outcome when all predictors are zero and ε_i is error (Field, 2011).

4.4 Effects of CO_2 Levels on Stomatal Density of British Samples

Step 3 is to determine whether changing CO_2 levels have affected stomatal density. This stage will also use simple linear regression (Equation 1) to model the relationship between past CO_2 levels and SD estimates obtained from British herbarium specimens of a range of ages. The CO_2 data that will be used derive from measured air bubbles trapped in an Antarctic ice core (AD 1740-1950) and atmospheric CO_2 concentrations directly measured from Mauna Loa Observatory in Hawaii (AD 1950-2000). All data were obtained from Beck (2007).

4.5 Effects of Both Climate and CO_2 Levels on Stomatal Density

Step 4 is to determine whether variation over time in stomatal density can be better explained by including climate factors in the explanatory model. The model developed in step 3 (see Section 4.4) will be extended by including those measures of past climate which were found to be significant during step 2 (see Section 4.3), creating a multiple regression model (Equation 2). Climate data will be obtained from documentary records available for public download (see Table 5 for data sources).

5. Results

5.1 Leaf Architecture of *S. cinerea*

SEM images show that *S. cinerea* leaves have a complicated wax coat, consisting of conical structures (see Figure 7). These 'conicoids' are known to be present on *S. alba*, *S. fragilis*, *S. triandra* and *S. pentandra* (Tomaszewski, 2004), but no literature was found reporting their presence on *S. cinerea*.

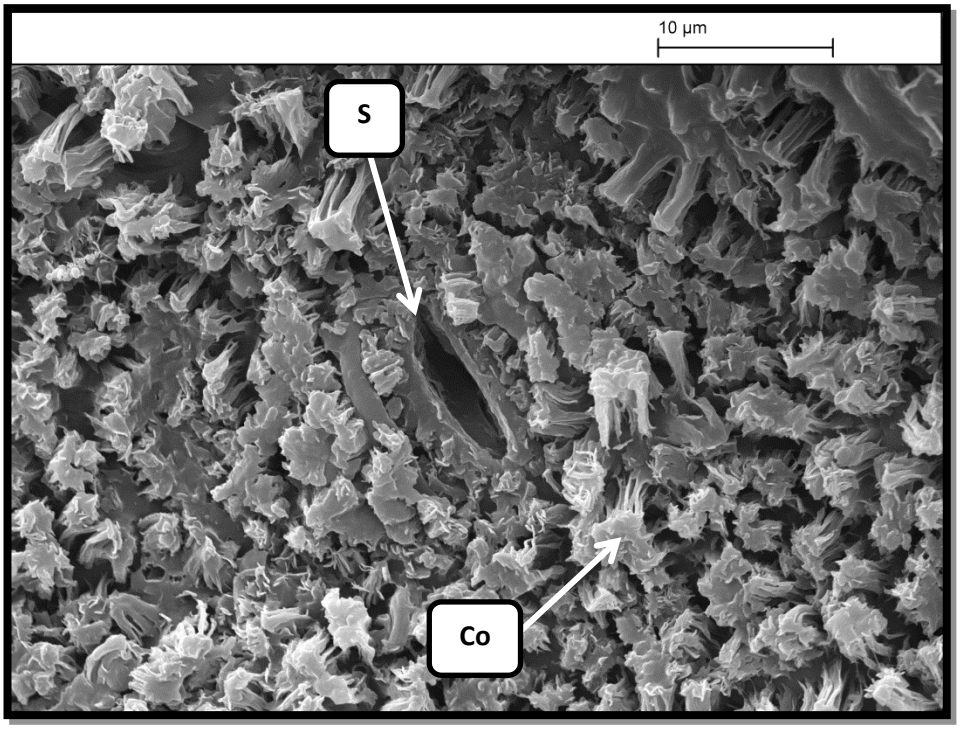


Figure 7: SEM image of a modern *S. cinerea* leaf collected from Muckross Peninsula (Ireland); taken at 750x magnification, scale bar represents 10µm. Note that *S* is a stoma and *Co* is the conicoid features.

In addition to conicoids, the wax has small crystalline features (see Figure 13, in Appendix 1), also seen in other *Salix* species (Chen *et al*, 2008; Gołębiowski *et al*, 2008; Gostin and Ivanecu, 2007). Other species possess spherical wax formations (Genet *et al*, 1997) but none were seen here. Although a combination of this complex wax structure and sunken stomata made it unlikely that a successful impression could be created of *S. cinerea*, non-destructive indirect methods of observation were tested anyway. This is primarily because such techniques do not destroy the leaves and are inexpensive. If such methods proved successful, then a larger data set could be collected to address the second research question. Such methods have worked effectively on some species of *Salix.* For instance epifluorescence microscopy has been used to count stomata on *S. herbacea* (Beerling and Rundgren, 1999; Beerling and Rundgren, 2000; Björck and Rundgren, 2003; Björck *et al*, 2007).

5.2 Methods Used

5.2.1 Non-destructive Indirect Observation

Acetate Peel (see Section 3.1.2)

No peels showing a clear image of part of the leaf surface were obtained using this method, probably due to the surface of the samples not being smooth. Beerling and Chaloner (1992) argued that the method is unsuitable for hairy specimens, and these findings support that.

Nail Polish (see Section 3.1.2)

Trials on dried *S. cinerea* leaves confirmed that the brittle nature of the leaf specimens meant that it was difficult to produce quality impressions of any great size. Experiments on both modern air dried *S. cinerea* and historical samples resulted in failures. In contrast modern air dried *Betula pendula* leaves provided clear replicas which illustrated not only the stomata but all of the surrounding epidermal cells, but getting impressions of large areas was still a problem. Although nail polish creates accurate representations of some leaf surfaces, only small areas can be a studied at a single time because the tension created during the removal process is too great for a large patch of varnish to withstand, causing stretching and tearing of the impression.

Specimen L18 (3rd leaf down from the top of a modern dried *B. pendula* branch) produced the best result with 100% of leaf cover successfully lifted; other samples produced impressions of 40-50% of the leaf at best and often less than that (see Figure 14, in Appendix 1). This equates to a similar size impression because L18 was 3.8cm long and 2.7cm wide, whilst for example sample L20 (3rd leaf down from another modern dried *B. pendula* branch) was considerably larger at 6.4cm long and 5.1cm wide. The optimum size for *B. pendula* peels is approximately 3cm by 3cm. Nail polish remains possibly the best option for obtaining impressions of a leaf's surface because it is quick, simple and inexpensive, but it is not suitable for *S. cinerea*, and may be destructive when used on herbarium specimens.

Nitrocellulose Lacquer (see Section 3.1.2)

Experiments suggested that impressions could be made of no more than 5% of the leaf at best. Separating the replica from the surface of the leaf was difficult, as the substance penetrated all of the surface's irregularities (i.e. micro-furrows of cuticular wax and hair fibres) and became stuck.

Nitrocellulose lacquer is less viscous than nail varnish, and it was anticipated that due to the lower viscosity, the chances of all of the smaller details being covered would increase leading

to a better impression. Coverage was improved, but the peel could not be removed from the leaf. If an effective method of removing the impression from the samples were discovered, this method could be valuable, as the aerosol container made for an even application and the lacquer coated the complex surface closely.

Cyanoacrylate Adhesive (see Section 3.1.2)

Peels were obtained using this method but they were not transparent enough for proper examination under a light field microscopy using an Olympus compound microscope at 400x magnification (see Figure 16, in Appendix 1). Diluting the solution with water did improve the quality of the peels a little. The larger the H_2O content, the greater the chance of shrinkage and curling of the impression and the peel was no more transparent than without dilution.

This may be a plausible method for a species with a less complex leaf surface architecture, but nail polish produces clearer peels. Although a larger area may be sampled using cyanoacrylate adhesive in a single peel, the level of detail is compromised. If a larger area did need to be sampled then with nail polish multiple impressions could always be made, thus providing a greater area covered, whilst retaining a high standard of detail.

Dental Material (see Section 3.1.2)

The first variety tested, Hiflex DIY Putty®, successfully replicated the vein network of *S. cinerea* but failed in recording any other detailed cell structure. Once the Hiflex mould is set, it takes on a rubber like quality which makes removing the nail polish impression easy due to its flexibility. Elite HD+®, a higher quality quick setting material used by professional dentists for taking impressions, proved less easy to work with, and did not record more detailed cell structure. In order to determine whether this was due to the complex leaf surface structures of *S. cinerea* (see Figure 17, in Appendix 1), impressions from *Betula pendula* and *Quercus robur* were also tried, with no better results.

The dental impression paste (Cavex Outline®) was more difficult and messy to mix and work than the putties. Often a gum-like effect occurred when attempting to remove leaf specimens, resulting in either poor quality or no replications at all. Putty alternatives cause less mess, have a longer 'working time' and provide more consistent results. Despite there being many examples of this method being used in the literature (e.g. Beerling and Chaloner, 1992; Black *et al*, 2002; Jackson *et al*, 2002; Perez, 2008 see Section 3.1.2), this method is found to be less effective than nail polish as well as being far more expensive. If any non-destructive indirect

observational method were suitable for the study species, then using nail polish would be the most appropriate method.

5.2.2 Destructive Direct Observation

Scanning Electron Microscope (see Section 3.1.3)

SEM produced high quality images, with excellent detail. This effectively illustrates the complicated wax microstructure and provided an explanation for all of the previously stated methods being relatively unsuccessful (see Figure 7 in Section 5.1, Figures 18, 19 and 20, in Appendix 1). Although this methodology is consistent and precise, it remains a destructive method and requires sacrificing a leaf. It is also considerably more expensive than all of the other methods. Furthermore only the SD could be counted using SEM alone, because the epidermal cells are obscured by a wax layer. If an effective method of wax removal could be applied to the leaf samples first, then SEM would be capable of providing SI data. The lack of SI data is due to limitations of the leaf, rather than limitations of the method itself. Overall this was the best method for obtaining stomata data from *S. cinerea*. This method provided SD counts ranging from 1 stoma (see Figure 18, in Appendix 1) to 49 stomata (see Figure 19, in Appendix 1) in a single 275µm by 175µm field of view (see Table 8, Appendix 2 for further results and Section 4.4 for statistical analysis of the counts). This equates to a range of 21 to 1021 stomata per millimetre square.

Epidermal Scraping (see Section 3.1.3)

The same cuticular masceration method that McElwain *et al* (1995) used to study stomata on fossilised *S. cinerea* was tested on herbarium *S. cinerea*. H_2O_2 is hazardous, however only a relatively diluted substance is required and as long as proper precautions are in place (fume cupboard, laboratory coat, goggles and gloves), the chance of serious incident are minimised. Another problem is that a single leaf may only be sampled once because this is destructive, so there is no room for error because a leaf cannot be resampled. An advantage however is that the entire leaf may be sampled at once. During experimentation with *S. cinerea* leaves, it was also noted that the timing is slightly different depending on the individual sample. This meant that it was sometimes difficult to get consistent results. Furthermore some areas of the leaves did not digest at the same rate as the rest of the leaves, resulting in small patches of the sample not being entirely transparent or free of the mesophyll tissue (see Figures 27 and 28, in Appendix 1). It is for this reason that this method was abandoned. Without time constraints this method may possibly be perfected in order to obtain a consistent set of results; however in this investigation this was simply not possible.

5.2.3 Destructive Indirect Observation

Wax Removal (see Section 3.1.4)

The presence of wax and leaf hairs has resulted in many of the cheaper, non-destructive methods of counting stomata not being effective. In an attempt to prepare leaves for taking impressions, various methods of wax removal were tested (see Section 3.1.4). All of the methods experimented with used chloroform ($CHCl_3$), as it was the most readily available material and had minimal health implications (providing a fume cupboard is used).

All of the $CHCl_3$ methods caused the wax to be partially digested. It was clear that small quantities of the wax had detached from the surface of the leaf. However, most of the wax present was not removed, but had in fact just been smeared across the surface. Some samples even had small bubbles of gas (see Figures 29 and 30, in Appendix 1) which had stuck to some of the remaining wax and the hair follicles; presumably this is gas that was trapped inside the leaf after the sample had been picked and pressed.

Further investigation is required before determining whether wax removal is an appropriate technique for this species. Although the experiments tried in this investigation did not successfully remove the wax cuticle, this does not mean that it is not possible for it to be removed using some other method. A longer duration chemical treatment, more agitation, more heat, or a combination of all three may prove to be more successful. Bevilacqua *et al* (2002) suggest that some Salix species have a thicker coat of wax in September than in March. Bearing this in mind, it may be worth treating leaves that were collected later in the year for slightly longer. More experimentation would be worthwhile if time were available.

5.2.4 Methods Summary

Most non-destructive indirect methods of observation were ineffective due to the leaf architecture and SEM proved to be the most successful method for obtaining SD data from *S. cinerea*. The main limitation of this method is that it is expensive (therefore restricting the amount of data that can be collected) and, due to the wax cuticle, still cannot provide SI counts. It relies on the assumption that a count of exposed stomata in a fixed area is a reasonable estimate of stomatal density across the leaf. These counts range from 1 stoma to 49 stomata present in a field of view of 275µmby 175 µm, or 21 to 1021 stomata per millimetre square.

5.3 Stomatal Density Data

5.3.1 Origin of Data to Be Analysed

The stomatal density data were obtained by systematically searching photographs of the leaf surface obtained via scanning electron microscopy (SEM). Stomata were counted in a consistently sized area of the edge and the centre of each leaf sample (see Section 3.1.3). The physiology of *S. cinerea* leaves made for challenging collection of data. As previously stated in Section 5.1, a thick complex cuticular wax (in the form of 'conicoids') obscures the stomata and hides the epidermal cell structure. Better results may potentially be obtained using a combination of a wax removal method and a peel technique, as the SI could be recorded instead of just the SD. Due to time constraints, enough experimentation of this method was not carried out and so the counts from the SEM images are the only data which can be statistically analysed.

Stomatal counts ranged from 417 to 21 (stomata per millimetre square) in the modern samples, and from 896 to 21 (stomata per millimetre square) from the full set of samples which represent a time span of 129 years. The means of each sample are presented in Table 8 (Appendix 2), along with the mean stomatal count for sample areas located at the edge and in the centre of each leaf (which are also displayed in Figure 8).

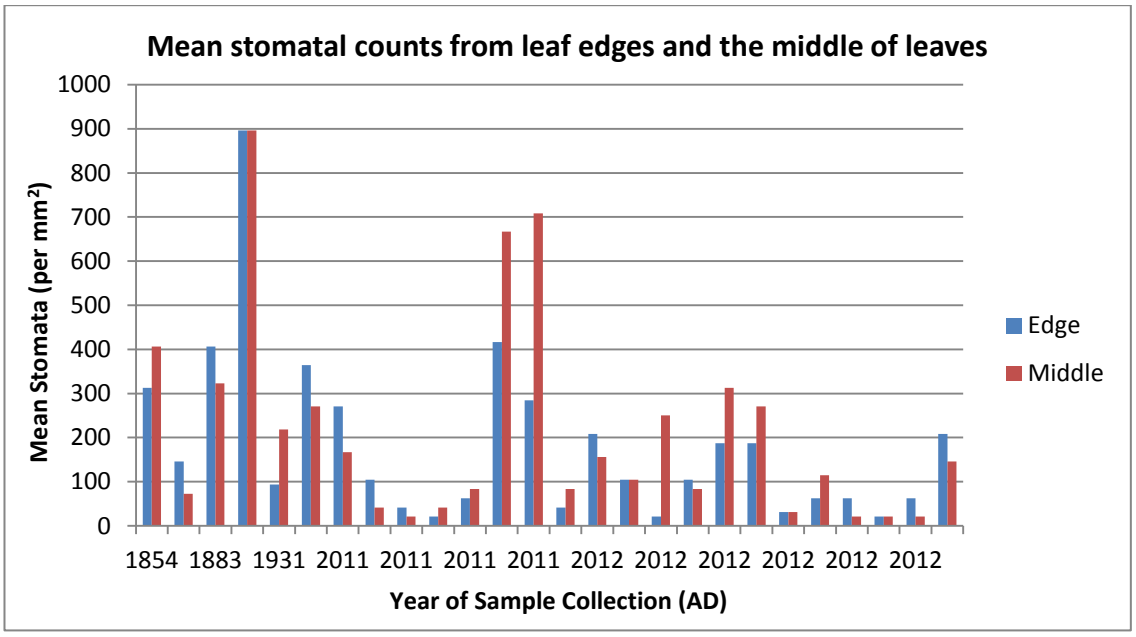


Figure 8: Mean number of stomata per millimetre square on the edge and in the centre of leaf samples collected from different periods in time.

5.3.2 Effects of Sampling Strategy on SD

Analysis of SD data from specimens sampled in different ways (see Section 4.3) shows that there is no statistically significant difference ($p > 0.05$) between the SDs obtained from the edge of the leaf or the centre of the leaf (H_1), pressed leaves or air dried leaves (H_3), and leaves in the same position but on different twigs from the same branch (H_4). However there is a statistically significant difference in SD ($p < 0.05$) when sampling leaves from different positions on the same twig (H_2) and leaves in the same twig position from completely different specimens of the same species (H_5) (see Table 6).

Hypothesis	Test Used	t Test Value	F Value	p Value	N	H_{0x}
H_1 (edge vs. middle)	T Test	0.897	-	-	35	Accept
H_2 (leaves on the same twig)	ANOVA	-	9.768	0.002	18	Reject
H_3 (pressed vs. air dried)	T Test	0.738	-	-	9	Accept
H_4 (leaves from same position, different branch)	ANOVA	-	2.887	0.066	17	Accept
H_5 (leaves from the same position on samples from different trees)	ANOVA	-	5.382	0.001	32	Reject

Table 6: Results of the hypotheses testing, using a 95% confidence level and deeming values with a p value greater than 0.05 as statistically insignificant.

5.3.2.1 Sampling Strategy for Studies of Effects of Climate and Time Differences

SD is not affected by drying method or position of the sampled area on the leaf, but the position of the leaf on the twig does affect the SD, therefore the sampling strategy adopted was to collect SD data from leaves taken from first year twigs to mitigate against this cause of variation. As H_1 (see Table 6) concluded that there was no statistically significant difference between stomatal counts obtained from the edge and the middle of leaves (see Figure 8), no distinction was made for further analysis of the data. Combining the SD of the leaf edges and centre allowed for an overall mean SD of each leaf sampled to be obtained (see Table 8, in Appendix 2).

6. Environment and Stomatal Density

Tables of the full set of ancillary climate data used in Section 6 are presented in Figures 32, 33 and 34 (in Appendix 3). The climate-related parameters chosen are: mean temperature of the spring (T), mean monthly maximum temperature of the spring (TM), mean monthly minimum temperature of the spring (Tm), mean monthly precipitation of spring months (PP) and mean monthly hours of daylight for the spring (L). The latitude of each modern sample was obtained by GPS reading (see Section 3.2). From daily temperature the length of the growing season (GS) for each location was calculated and may also be found in Table 12 (in Appendix 3). The method for counting the growing season is one that is standardized in the UK (Hulme and Mitchell, 2002) and may be defined as beginning after five consecutive days above 5°C and ends after five consecutive days below 5°C. It should be noted that only modern samples collected in 2011 have this data, as the length of the growing season cannot be calculated for 2012 yet.

The overall mean temperature and precipitation of the spring months (March-May) was calculated because heat and water availability both affect the rate of evapotranspiration rates of a plant during this key period of leaf development. All climate data that has been obtained is for the spring months because this is when the leaves are first growing and developmental triggers are set. Leaves will continue to grow, but it is the spring months which are the most important. The average maximum and minimum temperatures were also collected because they offer a measure of seasonality which is not shown by mean annual temperature. The length of daylight of the sample location was used as a proxy for light regime. These values were taken from recorded values of the nearest city; consequently average hours of daylight could be calculated for modern specimens only. The amount of daylight an area experiences does not account for the actual amount of light which a plant may utilise, in combination with the levels of precipitation however, inaccuracy (created by the presence of cloud cover) should be accounted for.

Table 13 in Appendix 3 contains the raw CO_2 data to correspond with the modern samples, which is also displayed in Figure 9 below. As mentioned in Section 4.3, half of the CO_2 data used were measured from trapped air bubbles in an Antarctic ice core (AD 1740-1950). The rest was directly recorded from Mauna Loa Observatory in Hawaii (AD 1950-2000). All data were obtained from Beck (2007).

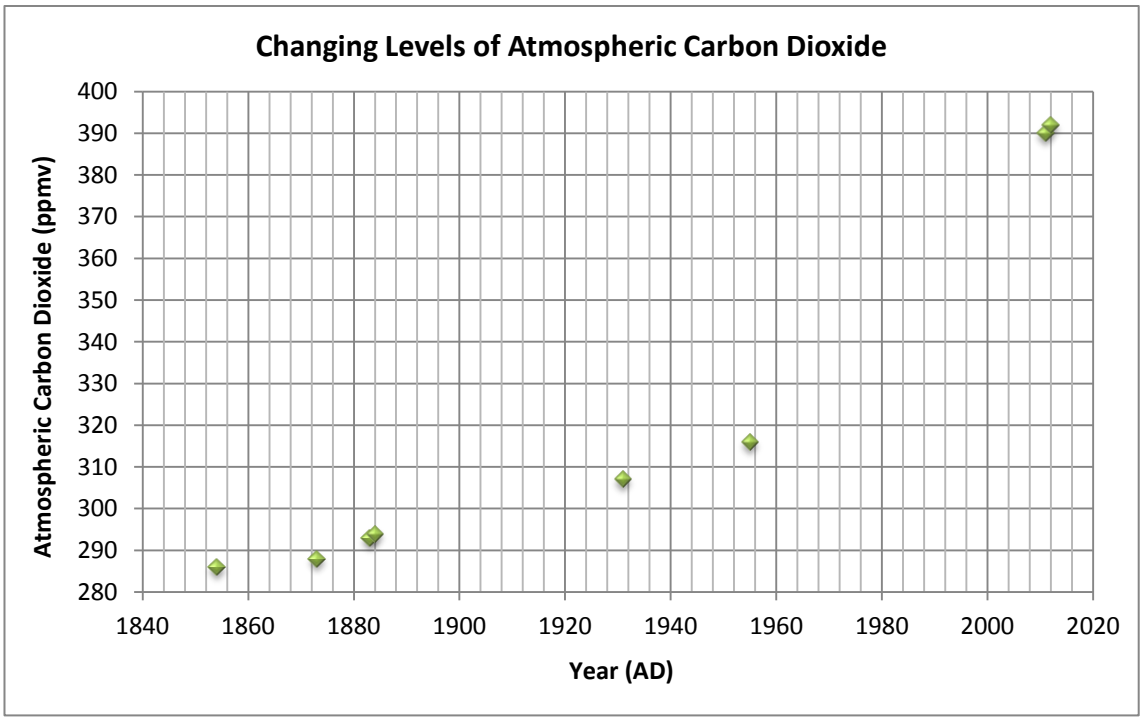


Figure 9: Changing levels of atmospheric CO_2 over time, using raw data from Table 13 in Appendix 3.

Previous research (see Section 2.2) suggests that both climate and CO_2 levels are possible controls on stomatal density. Most past studies however only investigate one factor or the other. This project aims to assess the significance of these contributing components in relation to observed variations in SD. By combining data of both climate and CO_2, a comparison may be drawn as to which factor is the more important influence on the SD (if a significant relationship exists).

6.1 Effects of Climate on SD (see Section 4.3)

6.1.1 Simple Regression Analysis on Modern Samples

Table 8 in Appendix 2 shows the mean SD counts and places they were obtained from. Table 7 summarises the overall mean, the standard deviation and the number of samples (N) collected from each geographical region. It should be noted that in this instance N refers to the number of first year twigs collected from an area. For example, a single twig was collected from three different trees. The mean is of all of the SD counts from the edge and the middle of each leaf tested from those three different tree samples and is therefore an overall representation not only of the entire leaf, but the entire geographical area. It is difficult however to state what the trends of spatial variation are using Table 7 (see next page) alone.

Sample Region	Number of Samples (N)	Mean	Standard Deviation
Ireland	3	231	166.71
Yorkshire	6	75	57.97
Staffordshire	6	156	66.94
Sweden	3	151	117.97
Germany	1	21	-
France	1	42	-

Table 7: Stomatal density in relation to geographical distribution of the sample source.

To determine whether the differences in the modern samples are statistically significantly different (and that spatial variation occurs) ANOVA was used. The results of which produced F = 1.700, P-Value = 0.199 and F Critical = 2.968, with a sample size (N) of 21. As $p > 0.05$ it may be concluded that there is no statistically significant difference between the modern European samples, therefore no spatial variation exists. This is not the result that was to be expected as climate is thought to have a significant input and so one should expect to find a difference in stomatal abundance based on the geographical location of the sample. Such results could be due to a small dataset. Another possibility is that climatic controls are a significant contributor but may be less significant for some individual *S. cinerea* plants. Some individuals will be naturally more robust than others, so will not be affected by climate as much. This is reinforced by Section 5.3.2, which has previously confirmed that separate samples are significantly different.

Simple regression analysis was used to test whether each climatic variable makes a statistically significant contribution to the SD of the modern European samples; the results are outlined in Table 14 (in Appendix 4). Each predictor was individually input into a regression model using the 'forced entry' method in SPSS. The stomatal data that were used were the overall mean values of SD for each individual leaf sampled, since there is no significant difference between the edge and the middle of the leaf (see Section 5.3.2). The resulting change table (Table 15, in Appendix 4) may give an impression of the effect each climatic control has on the stomatal abundance, based on the SPSS models in Table 14 (Appendix 4), if those models were statistically significant. The table suggests that the average precipitation of the spring months results in the largest amount of change in SD. For every 9.90mm (one standard deviation) increase in precipitation, the density would increase by an additional 1.79 stomata. This is calculated by multiplying the standardised Beta value of average precipitation (0.366) by the standard deviation of the stomatal density constant (4.89).

In addition to the potential resulting change, the Pearson's Correlation Coefficient was calculated in SPSS, in order to analyse the relationship between each predictor and the overall

mean SD on an individual basis (see Table 16, in Appendix 4). It shows that there is no significant correlation between any climatic variable measured and the overall mean SD of the modern samples. These values are only true when each predictor is input into a simple regression model separately.

6.1.2 Simple Regression Analysis on Modern English Samples

Section 6.1.1 concluded that an accurate model could not be made of each predictor on an individual basis. These results were unexpected, as climate is thought to have been one of the main drivers in the SD. To make a better assessment of the influence climate has on SD, a further model was developed using only the data of the modern English samples. The same 'forced entry' method (as used previously) will be applied to each predictor separately. Factors such as light and latitude will not be considered, as the samples were obtained from a relatively close proximity. By excluding these variables a clearer view of the effect of temperature and precipitation may be established. Other errors may be reduced because continentally has been eliminated and there is a smaller spread to focus on. Results of the simple regression model are presented in Table 17 (in Appendix 4).

The outcome of these regression models is a stark contrast to the models of all modern samples combined (Table 14, in Appendix 4). In Table 17 (in Appendix 4) the R^2 is far greater for each predictor and the significance levels have been improved. The only problem is that each of the models claim that the predictor accounts for 66.4% of variation in the outcome ($p = 0.014$). To understand these results better, a model combining all of the climatic variables may be necessary. To understand the relationship between the climatic controls and the constant individually, a resulting change table and a correlation table have been included, following the same principles as Figures 38 and 39 (in Appendix 4). Table 18 (in Appendix 4) shows a negative relationship existing between the average temperature and stomatal abundance; whereas a positive one is maintained between the average precipitation and SD. Pearson's Correlation Coefficients in Table 19 (in Appendix 4) reiterate these relationships.

6.1.3 Multiple Linear Regression Analysis on Modern Samples

All climatic factors were then included in a multiple regression analysis to explore how well stomatal density could be explained as a response to climate factors, and which factors were found to be statistically significant in a combined model. As no previous research of a similar nature has been completed on this species before, a hierarchical entry of data in SPSS was considered to be inappropriate. There was no evidence to suggest which variables are the most important, therefore no basis for making such choices. A stepwise method was not used

to avoid the suppressor effect, of missing predictors which predict the outcome (Field, 2011). For these reasons the method of regression chosen was forced entry which is considered to be the most reliable in replicating results (Cassidy and Studenmund, 1987). As with the simple regression, the method was applied to the overall mean values of SD for each individual leaf sampled.

Results are summarised in Table 20 (in Appendix 4), and show that a representative predictive model may not be built for the SD from the climatic parameters that were tested. This is denoted by the significance level of the R^2 value ($p = 0.207$). It should however be noted that no single parameter is significant alone because each individual parameter has a significance level greater than 0.05. If no single parameter can be isolated as the most important contributing factor towards the SD count, then it may be assumed that either a parameter that has not been included in this model (because the data was not available to include, or a relationship was not predetermined to exist and so was not thought necessary to obtain data of that influencing factor), or else a complicated relationship exists between all of the predictors which results in a significant contribution to the outcome of SD. This is reinforced by single factor regression which was carried out with each predictor individually.

All of the predictors in Table 20 (in Appendix 4) combined would account for 37.3% ($p = 0.207$) of the variation in SD if the model was statistically significant, leaving 62.7% of the variation unaccounted for. If the average length of daylight hours of spring months was included in this model then 5% more variation may be accounted for and a further 4.2% in addition to that if latitude of the sample was also included. However those models may be considered to be less effective predictive models as the probability of the results occurring due to chance is far greater, with $p = 0.317$ (including light) and $p = 0.353$ (including light and latitude). The results of these models may be found in Figures 44 and 45 (Appendix 4). Such unaccounted variation in the outcome may be due to factors which were not included in this investigation (because of data availability) like light intensity, atmospheric humidity and soil moisture; or CO_2 is the most influential control on SD.

As Table 20 has proven to be insignificant, a model was made with all of the climatic predictors run together on the data of the modern English samples only. This is because when all of the modern stomatal data was included, the simple regression model was not an accurate representation of the predictors' influence, but for the English samples alone they were. The outcome of multiple regression analysis on modern English samples was less successful; with the model excluding all variables except average precipitation of spring months (see Table 22,

in Appendix 4). Consequently this model offers little additional knowledge at this point. A greater contribution will probably be made by the model with an increase in sample size, as N = 8. The restricted sample size equates to a limitation in conclusions that may be drawn.

Conclusions that may be drawn at this stage, with these results, show that there is no statistically detectable geographic variation in SD; however a predictive model can be created based on climatic factors, which suggests that some aspect of the climate is a major influence in the observed difference. It is possible that the reason that there was no statistically significant difference between geographical locations is because more examples from each location are needed, as H_5 confirmed that there are differences between individual trees. Furthermore some climatic controls (such as average precipitation) do have a statistically significant impact on the SD of *S. cinerea*; however inconsistencies occur between certain models. This is most likely due to a small sample size and a complicated interconnecting relationship between the individual controls. To address the next project aim an assessment of the influence of atmospheric CO_2 on SD will be made, to determine whether more variance may be accounted for by that predictor.

6.2 Effects of Variations in CO_2

Before being able to determine whether CO_2 has a statistically significant effect on SD, the aim of understanding temporal variation must first be addressed. Data obtained from English samples only have been analysed in this section, working under the assumption that CO_2 is the primary control and that any climate effects are negligible. Figure 10 (see next page) demonstrates that there is a decrease in mean SD; the more recent the sample was collected. Pearson's Correlation Coefficient between the year of collection and SD is -0.532 ($p = 0.031$). When inputted into a simple linear regression model in SPSS R^2 is 0.283 ($p = 0.061$), confirming that whilst SD has altered over time, time itself is not a contributing factor.

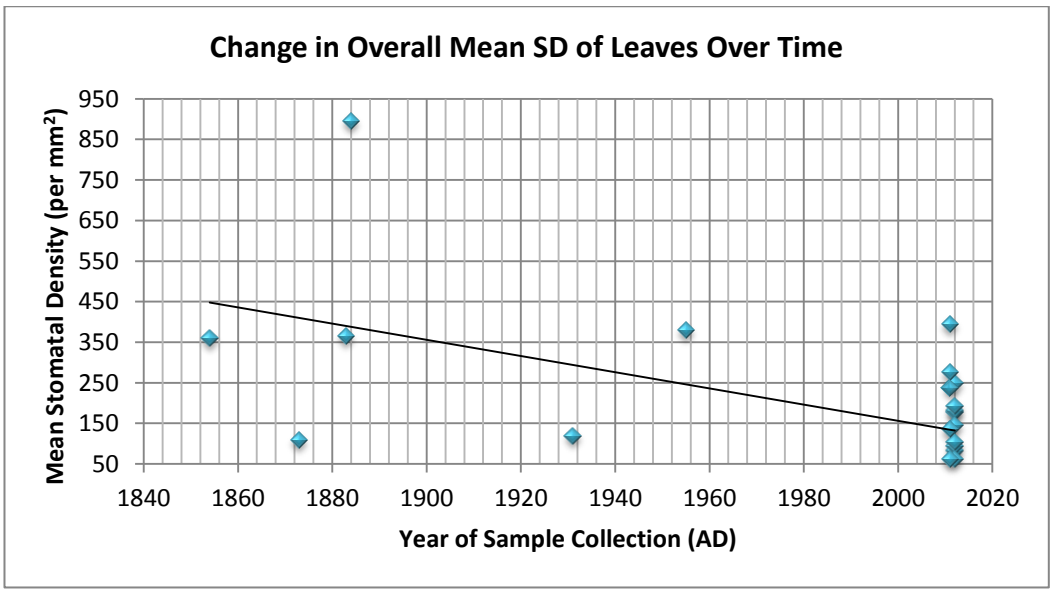


Figure 10: A scatterplot of the relationship between mean stomatal density and temporal variation.

Simple linear regression analysis between SD and atmospheric CO_2 was carried out in SPSS, on English samples only (including the modern English samples amongst the herbarium collection). Table 23 (in Appendix 4) shows that in this model a mere 2% of the outcome are accounted for by CO_2; an unexpected result for what is considered in the literature as a major contributor. This may be because the data set is relatively small. As suggested by Table 23 (Appendix 4), CO_2 alone is not a statistically significant contributor to the SD of *S. cinerea*. The results of Pearson's Correlation Coefficient in SPSS confirm a negative relationship between the predictor CO_2 and SD (R^2 = -0.143), but this should be disregarded as p = 0.274. It may be that although atmospheric CO_2 is not a statistically significant control in this model, if the data set were larger, it may be more influential. Alternatively it may be that CO_2 is only significant whilst in combination with contributing climate factors. It is not yet clear at this stage; Section 5.3 will help to determine whether a combined climate and CO_2 model is more effective at predicting the effect each predictor has on SD.

6.3 Effects of Both Climate and CO_2 (see Section 4.4)

For this stage of the analysis of all of the data for the herbarium and modern English samples was run in a multiple regression analysis model using CO_2 and climate variables. The results of this test show that 83.1% of the output (SD) is accounted for by the inputted predictors, leaving only 16.9% unaccounted for (Table 24, in Appendix 4). This is a substantially greater percentage than those from analysis run in Sections 6.1 and 6.2. Contrastingly when the same analysis was run on the modern European and modern English samples, the proportion of variance accounted for is much less, at 37.8% and the insignificance is far greater with p = 0.317 (see Table 25, in Appendix 4). A small amount of error in the second model may be as a

result of the exclusion of the length of growing season (which could not yet be calculated for the modern samples).

Examples of the resulting change in the stomatal density were calculated for all of the predictors, including only English samples (Table 24, in Appendix 4) and including all European samples (Table 25, in Appendix 4). Although the tables show that the predictors have different effects on both data sets, this may be due to the model producing different Beta values. It should also be noted that Table 26, (in Appendix 4) is of greater value due to the numerical examples being based on the English model which has historical data included and is consequently a more accurate representation of the relationships that exist between SD and its contributing factors.

It may be concluded that whilst various measurements of temperature, precipitation, the length of the growing season and the level of atmospheric carbon dioxide do account for some of the variability in stomatal density of *S. cinerea* leaves; the collection from different locations and different individual trees is also a very important contributor. It should be noted that much of the variability remains unaccounted for and these variables are not as influential as initially anticipated. This is probably because these results are affected by the small size of the data set. Comparing all of the models from Sections 6.1, 6.2 and 6.3, it would appear that the model which is the most effective at explaining the variance in SD would be the combined climate and CO_2 model run on the English samples, modern and historical (see Table 24, in Appendix 4). This is because the predictors account for 83.1% of the outcome, a larger amount than any of the other models, whilst retaining the best possible significance level ($p < 0.001$). If such an accurate model may be created for the English samples tested, then theoretically an equally 'successful' model may be made for the European data, if more samples could be obtained (including historical specimens). Unfortunately the data that is currently available is not sufficient to support or disprove any of the previously outlined hypotheses concerning influences on stomatal abundance with any great level of certainty. The English data however was considerably more adept at confirming that a combination of temperature, precipitation, length of growing season and atmospheric carbon dioxide significantly alters the stomatal density of *S. cinerea* samples collected from various points of time, from across England.

7. Discussion

7.1 Complications Concerning the Methodology

SEM images have confirmed that the surface of *S. cinerea* leaves has a complex protective structure including hairs and a multi-layers wax. Not only does this prevent a count of the stomatal index (SI), it also raises concerns about whether some stomata have been obscured and consequently have not been included in the SD total. If so, what percentage of stomata has been missed? How does this affect the overall results? Would the current trend be altered in any way? One assumes that the stomata will not be covered by wax when alive, or else they would not be able to function. What is clear from these images however is that some samples have a thicker wax coating than others. For instance, a specimen collected from Germany was incredibly waxy and hairy in comparison to a sample collected from Sweden. Does this mean that the SD count of the German sample is low because it has more stomata which remain out of sight, or is the SD count an accurate representation of the sample and is low because the plant has naturally adapted to have less stoma? Either theory is possible, in order to determine which is more likely more research needs to be carried out on developing an effective wax removal method for these leaves.

Once a more effective methodology has been developed, the complications of examining *Salix* may then be adequately addressed. For this investigation all forms of 'pussy willows' have been grouped together and treated as an aggregate because of their similar distributions and genetics (see Section 3.2). Further research should attempt to disentangle all of these species and collected data from them individually. This would not be an easy task as *S. cinerea* and *S. caprea* hybridise freely. It would also be interesting to discover whether there is a statistically significant difference between the two subspecies of *S. cinerea* (ssp. *oleifolia* and *cinerea*). If there is, additional error would have inadvertently been introduced to this particular study, once again highlighting the difficulties of analysing this species. In light of this research, it may be concluded that more work is needed to confront the questions of the taxonomic organisation of willow; in addition to developing a more effective method of obtaining stomatal counts from these complex leaf surfaces.

7.2 The Leaf Surface of *S. cinerea*

The leaf surface traits noted in *S. cinerea*, such as a protective wax layer and dense hairs, are typically a trait of Mediterranean vegetation. Pubescence can partially protect the leaf from ultraviolet-B radiation damage (Drossopoulos *et al*, 1998). Lovelli *et al* (2012) suggest that in the Mediterranean area an expected temperature rise and water deficit will not be

compensated for by stomatal closure. *Olea europaea* (olive) trees in free-air CO_2 enrichment (FACE) facilities have displayed a decrease in SD under elevated CO_2 conditions (Minnocci *et al*, 2001). A limitation of this study is that other environmental conditions are not considered in accompaniment to CO_2, such is the case with the majority of other research on the species (Minnocci *et al*, 2002). Potentially this could result in misleading conclusions. For example the FACE experiments do not consider the levels of nitrogen (N) in the leaves, which has been proposed to reduce water-use efficiency in *O. europaea* under drought conditions (Díaz-Espejo *et al*, 2006). This is possibly an important relationship that needs to be investigated further if, like Lovelli *et al* (2012) claim, a water deficit will occur in the Mediterranean where *O. europaea* predominantly grow.

Whilst this may be a problem in arid climate zones, *S. cinerea* is a northern plant, of a wet, temperate climate and so is not likely to be affected in the same way. The most northerly distributed willow is *S. arctica* (Artic Willow); a species whose leaves are slightly glaucous, with long pubescence (Klinkenberg, 2012). In this instance these leaf traits are certainly not an adaptation to a warm climate. It is likely that this is, as Tomaszewski (2004) suggests with other *Salix* species, a protection against insects, fungi and diseases. Possibly a more appropriate explanation, considering moulds thrive in damp conditions.

7.3 Limitations of the Data

The results of this investigation have confirmed that for *S. cinerea* at least CO_2 is not the only statistically significant factor related to stomatal abundance and therefore interpreted as a cause of the variation seen. The best model developed from this data, in Table 24 (in Appendix 4 and discussed in Section 6.3), suggests that for *S. cinerea*, SD is positively correlated with mean spring temperature, mean spring precipitation and length of growing season increases. From this study the extent that each contributing factor has on *S. cinerea* is unclear, due to the high instability of numerically analysing a small data set.

Improvements could be made to this investigation if more herbarium samples could be used. H_5 (see Sections 4.3 and 5.3.2) suggests that there is a significant difference in SD between different trees growing in the same location, so the developed model could be strengthened by including data from more specimens from past years. It would be interesting to discover whether additional samples would reinforce the current trends. This would be possible with more funding available, as there are multiple samples that were collected from the same year. An improvement that cannot be made however would be to increase the number of samples tested from different years. Unfortunately this is not possible, as the herbarium collection is

not that extensive and samples were not collected at regular intervals, which have left large gaps in between some of the data points in the models developed. If more samples from the same year were tested, then perhaps some error that may occur due to these gaps may be reduced. This would be possible by assessing material from more herbaria. It may also help to address whether the difference between different trees is more significant than the difference that occurs due to the climatic conditions that a sample is subjected to.

The most ideal method of data collection for a study such as this one would be to sample an individual tree (or two individual trees from contrasting areas) and collect climatic data on site, factors such as soil moisture and light intensity could then be measured directly. Alternatively these factors could be measured during the development of the leaves in an experimentally controlled greenhouse environment. Shifting the emphasis of this study to focus on one (or possibly two) individual tree(s) would reduce the amount variability and produce a more 'stable' set of results. This investigation suggests that it not possible to effectively test multiple lines of variation with so few sample points. Concentrating on one specimen will allow for the collection of more easily comparable data points. The major limitation of this would be that it is still depending on the same methodology that has been previously used in this investigation. This is not idyllic as once again the stomatal density, not the preferred stomatal index, would be counted. Furthermore if the data are obtained from images using a SEM, only small areas of those leaves will be analysed because it is expensive and may be difficult to find funding for. Developing a method using a combination of wax remove and making a surface replicating peel would be preferable; providing a cheaper alternate method and enabling a larger area to be counted, giving more accurate estimates of stomatal abundance.

The final limitation is that climate data used were not always from the immediate vicinity of the sample location, as this was not possible. It should however be noted that all of the sites were not near mountainous terrain and so the climate data should be fairly representative of local conditions. Some error that may have occurred may be as a result of the discrepancy between the different sources of said climate data. This also could not have been avoided, as with ever improving technology weather data are more advanced, as such digital devices were not available during the 1800s, so the historical climate data is likely to be more inaccurate.

7.4 Further Work

This research supports earlier findings that species response to climate change will be individualistic, and that changes in SD during acclimation responses are not solely a response to CO_2 levels, but also climate parameters. Implications that this research has in regard to

environmental studies, is that more work should consider not only the relationship between the frequencies of stomata present in relation to an altered climate, but should analyse the limitations of each individual species. It would be useful to attempt to determine what the thresholds of different species are; this would provide a better idea of how climate change will affect the flora. In order to effectively do this however hundreds of samples would need to be collected to test multiple variations, which are not always possible to obtain or financially viable. An alternate line of investigation would be to collect a larger number of samples from an individual tree, or two trees from contrasting climates; as more rigorous within-tree testing is required, especially in regards to the different subspecies of *S. cinerea* and their hybrids.

8. Conclusion

The first objective was to develop the methodology used to obtain stomatal abundance data from S. cinerea. This objective can be divided into two sections: the first relates to how the SD counts may be physically obtained (i.e. what methods are available) and the second refers to developing a sampling strategy (i.e. how the methods can be applied).

In regards to the first part of this aim, it may be concluded that more work is required in order to obtain a more effect method of acquiring stomatal density data. This is because the only method that provided consistent results was using a scanning electron microscope (SEM); whilst being capable of creating highly detailed images of the leaf surface, is very expensive and revealed complex wax micro-structure. The physiology of the leaves surface rendered the more desirable non-destructive indirect methods of observation ineffective (see Section 5.2.1). A further problem with the wax layer is that even SEM cannot provide SI counts because the epidermal cells are obscured. Potentially it may also be concealing some stomata, which would make the current SD data obsolete. To determine whether this is the case, further experimentation with wax removal methods, or else an adaptation of cuticle maceration is required. Due to difficulties in developing an effective but affordable method, the data set that was analysed was relatively small.

The second part of the first objective was to discover whether leaf sampling affects the stomatal abundance data. Results of this research show that within-tree differences do occur in SD counts of *S. cinerea* (see Section 5.3.2 and Table 6). Statistical analysis confirms a significant difference in SD ($p < 0.05$) when sampling leaves from different positions on the same twig (H_2), as well as leaves in the same position from completely different specimens of the same species (H_5). There was no statistically significant difference ($p > 0.05$) between SD counts obtained from the middle and the edge of the same leaf (H_1); nor was there between leaves in the same position but on different twigs from the same branch (H_4) though. Different leaf treatment methods (air drying or pressing, H_3) did not result in a statistically significant difference in SD either. Subsequently the sampling strategy adopted was to collect SD data from leaves taken from first year twigs, so that the other objectives may be achieved without error created by within-tree variation.

The second main objective was to investigate spatial variation of stomatal abundance at present day in relation to climatic factors. ANOVA suggests that there is no statistically significant difference between the mean SD of modern samples from different locations in NW

Europe. It should be noted that N was only 21 and so ideally more data is required to fully address the questions raised by the second aim. It may however be that a variation in climate alone is not enough of a controlling factor to cause a statistically significant difference to occur in SD of *S. cinerea*.

The final main objective was to investigate temporal variation of stomatal abundance in relation to both climate and atmospheric CO_2 levels. Multiple models were developed showing the relationship between SD and CO_2 and climatic factors. The most 'successful' of the statistical models was based on data obtained from English samples collected from different time periods, thus have been subjected to a varying climate and atmospheric CO_2 levels. This model suggests that the SD is affected by a combination of temperature, precipitation, length of growing season and atmospheric CO_2. There is no linear relationship between SD and time, although this data does show that the number of stomata generally decreases the more modern a sample is. Simple linear regression shows that there is no statistically significant relationship between SD and time, suggesting that whilst there is temporal change in SD; time itself is not a contributing factor.

In summary, this investigation shows that there is no single causal factor statistically significantly correlated with the SD of *S. cinerea*, but a model combination both atmospheric composition and climate factors can be constructed which explains the observed patterns raises questions about the accuracy of similar model developed for other species which have only considered single casual factor.

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Appendix 1

A SEM image of the abaxial side of Leaf 7 from Sample Trentham, with enlargement of wax structure

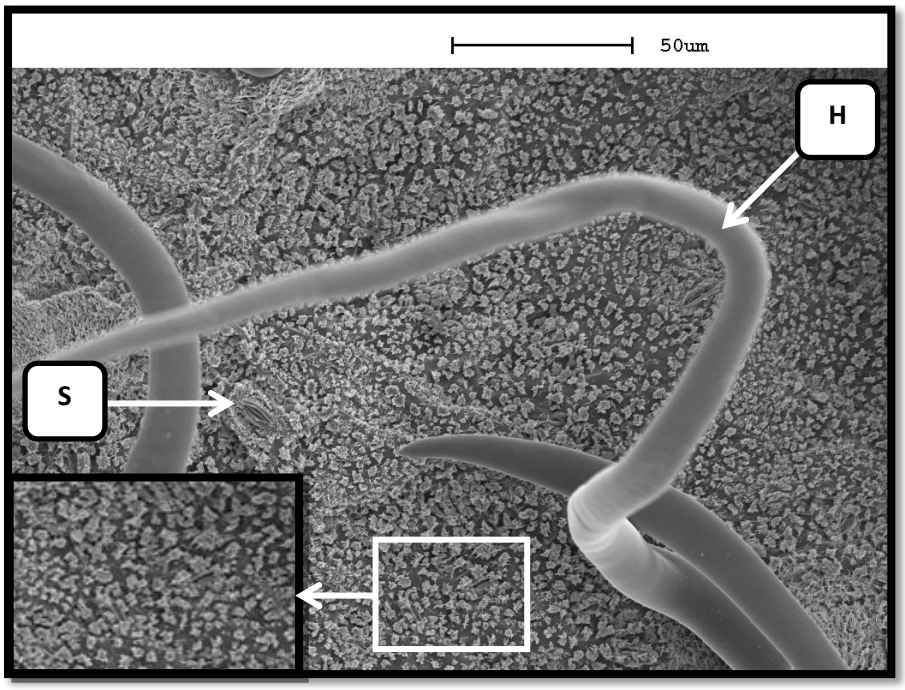

Figure 11: SEM image of a modern *S.* x *reichardtii* leaf collected from Trentham (Staffordshire); taken at 500x magnification, scale bar represents 50µm (but is not applicable to the enlarged image in the corner). Note that *S* is a stoma and *H* is a hair follicle.

A SEM image of the abaxial side of Leaf 23 from Sample 17158, with enlargement of wax structure

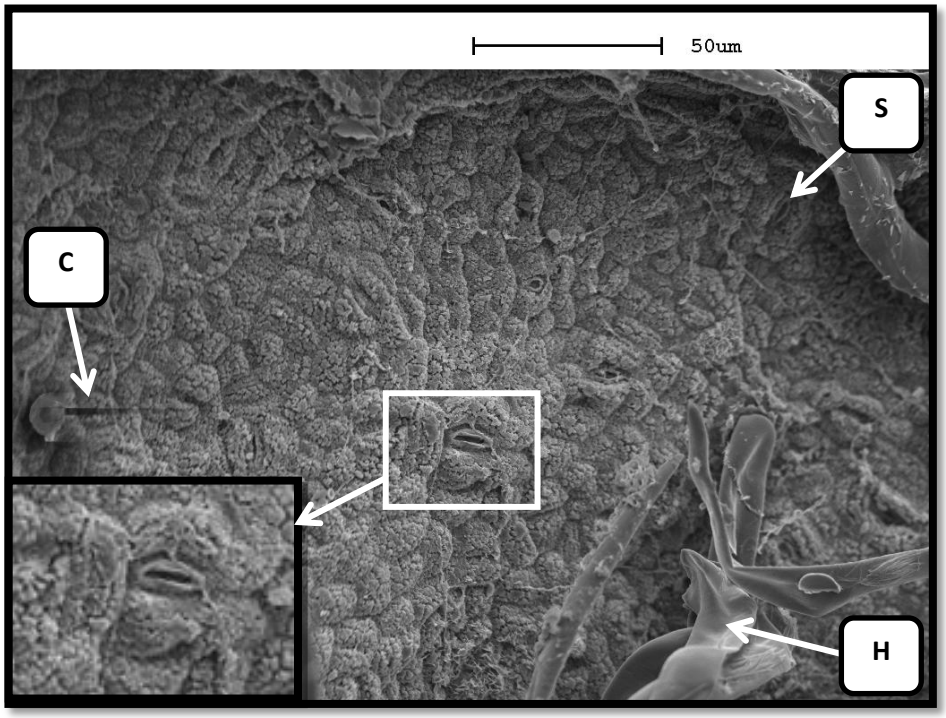

Figure 12: SEM image of a herbarium *S. cinerea* ssp. *oleifolia* leaf collected from Trentham (Staffordshire) in 1873; taken at 500x magnification, scale bar represents 50µm (but is not applicable to the enlarged image in the corner). Note that *S* is a stoma, *H* is a hair follicle and *C* is a 'charging' effect.

Appendix 1 continued

A detailed SEM image of crystalline wax features on the abaxial of Leaf 1 of Sample SS4 (17165a)

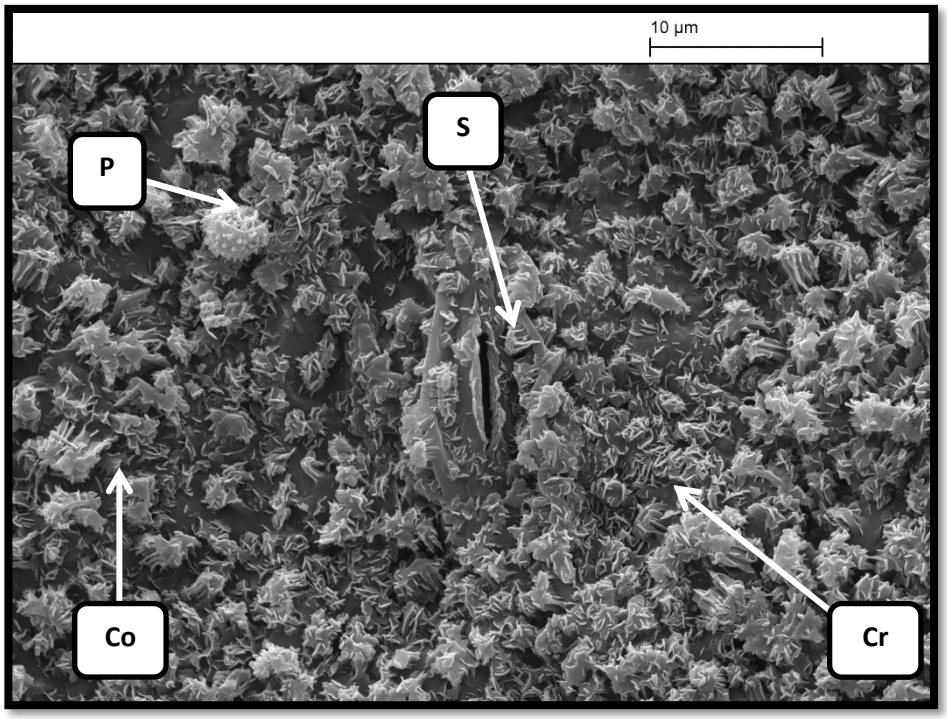

Figure 13: SEM image of a herbarium leaf (from 1884) of *S. cinerea* collected from Pool Hall Pool (Staffordshire); taken at 750x magnification scale bar represents 10µm. Note that *S* is a stoma, *Cr* is the crystalline features, *Co* is the conicoid features and *P* is Asteraceae pollen.

An image of a nail polish impression of the abaxial side of Leaf 26 of Sample Betula

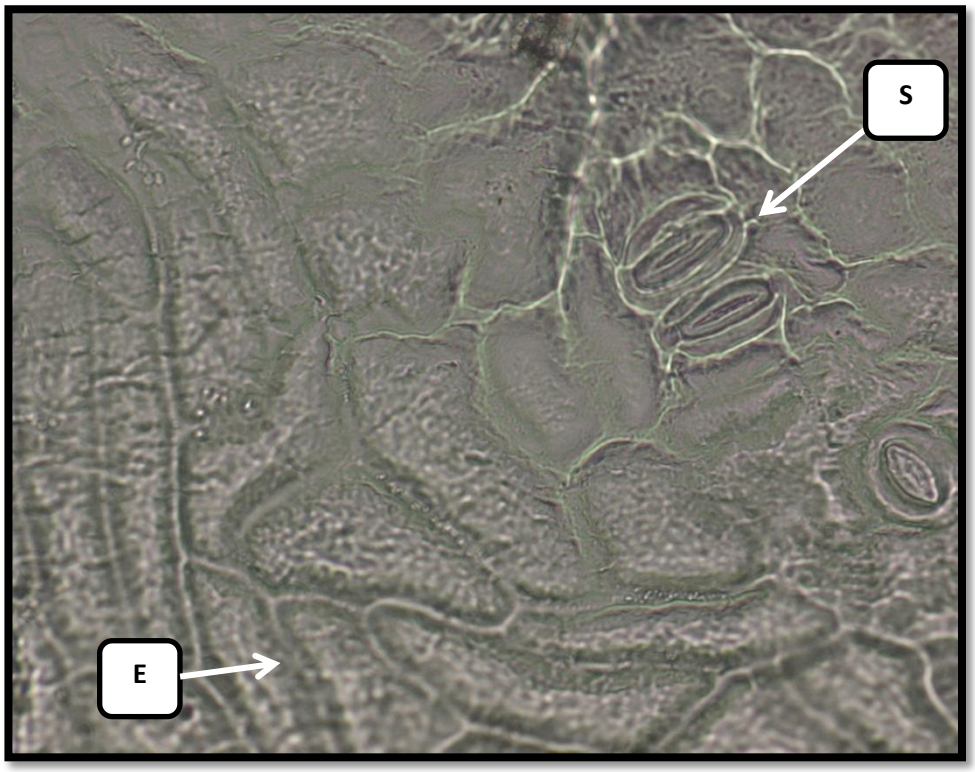

Figure 14: Nail polish impression of a modern *Betula pendula* leaf; image taken at 400x magnification, using an Olympus compound microscope. Note that *S* is a stoma and the *E* is an epidermal cell.

An image of a nitrocellulose lacquer impression of the abaxial side of Leaf 40 of Sample S1 (T4)

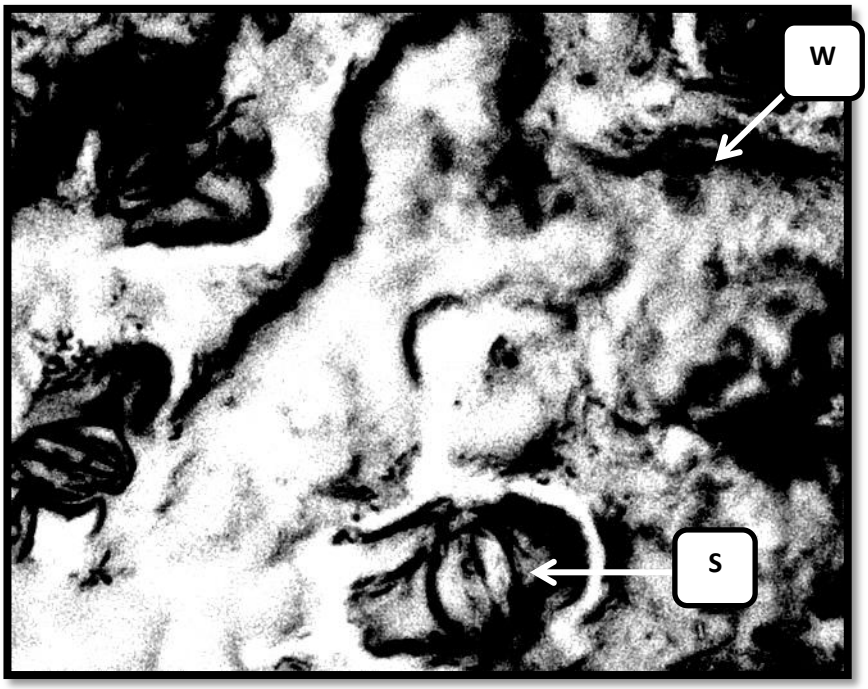

Figure 15: Nitrocellulose lacquer impression of a modern *S. x sepulcralis* leaf, image taken at 400x magnification, using an Olympus compound microscope. Note that *S* is a stoma and *W* is the waxy surface.

An image of a cyanoacrylate adhesive impression of the abaxial side of Leaf 13 from Sample S1

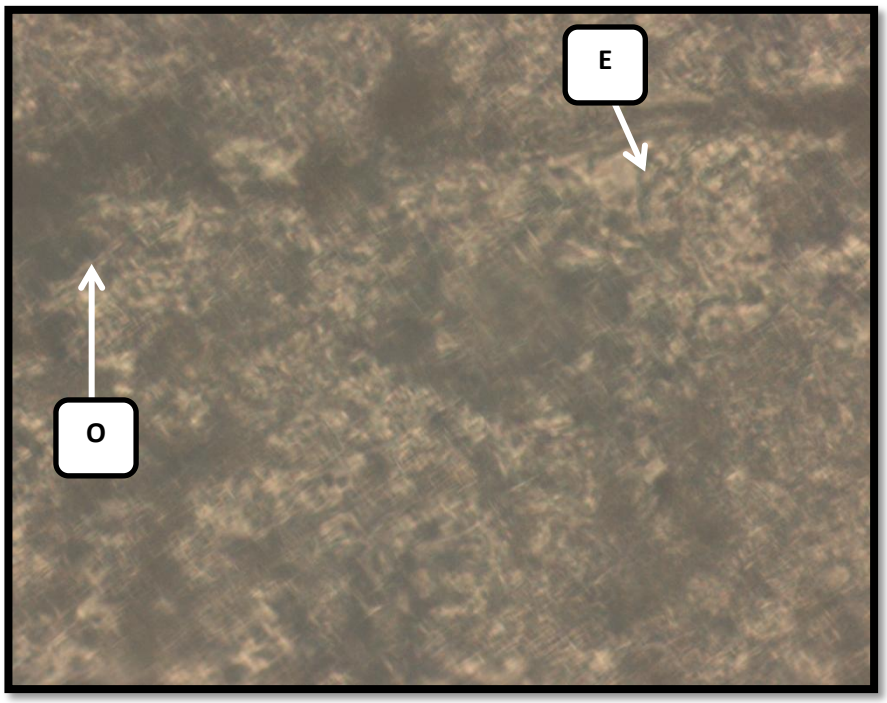

Figure 16: Cyanoacrylate adhesive impression of a modern *S. x sepulcralis* leaf, image taken at 400x magnification, using an Olympus compound microscope. Note that *E* is the outline of a possible epidermal cell and *O* is where the peel is not transparent enough to properly visualise.

Appendix 1 continued

An image of a nail polish impression from a mould (using dental material) of Leaf 4 from Sample S1

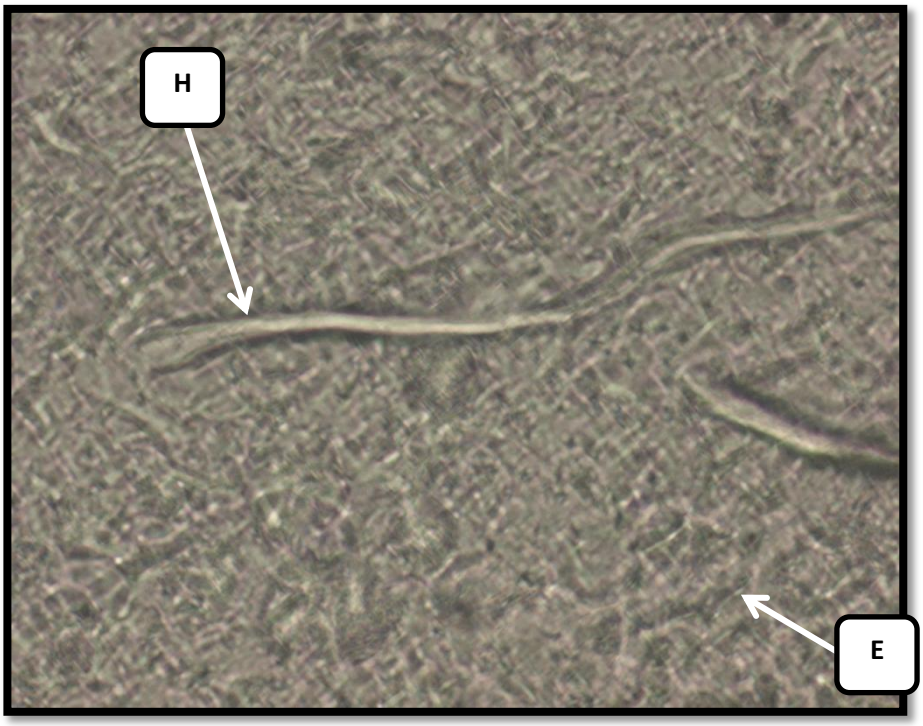

Figure 17: A nail polish impression from a dental material mould of a modern S. x sepulcralis leaf, image taken at 400x magnification, using an Olympus compound microscope. Note that *H* is the outline of a hair follicle and *E* is an epidermal cell.

A SEM image of the abaxial side of Leaf 16 from Sample B217

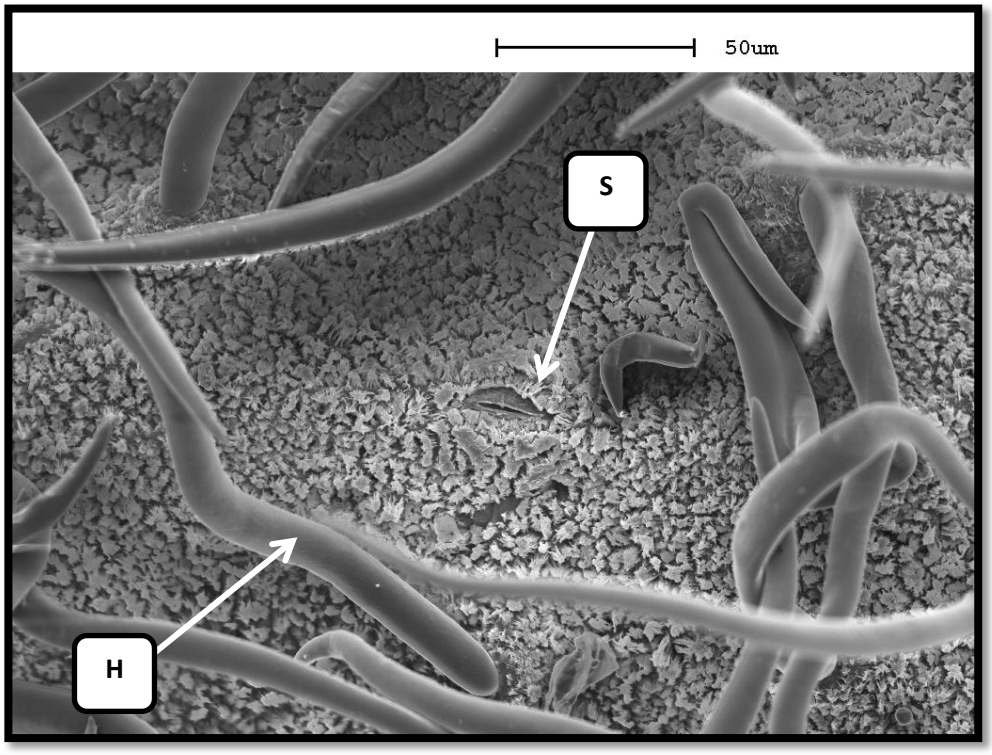

Figure 18: SEM image of a modern *S. cinerea* leaf collected from Schwenningen Moos (Germany); taken at 500x magnification, scale bar represents 50µm. Note that *S* is a stoma and *H* is a hair follicle.

A SEM image of the abaxial side of Leaf 2 from Sample 17165a

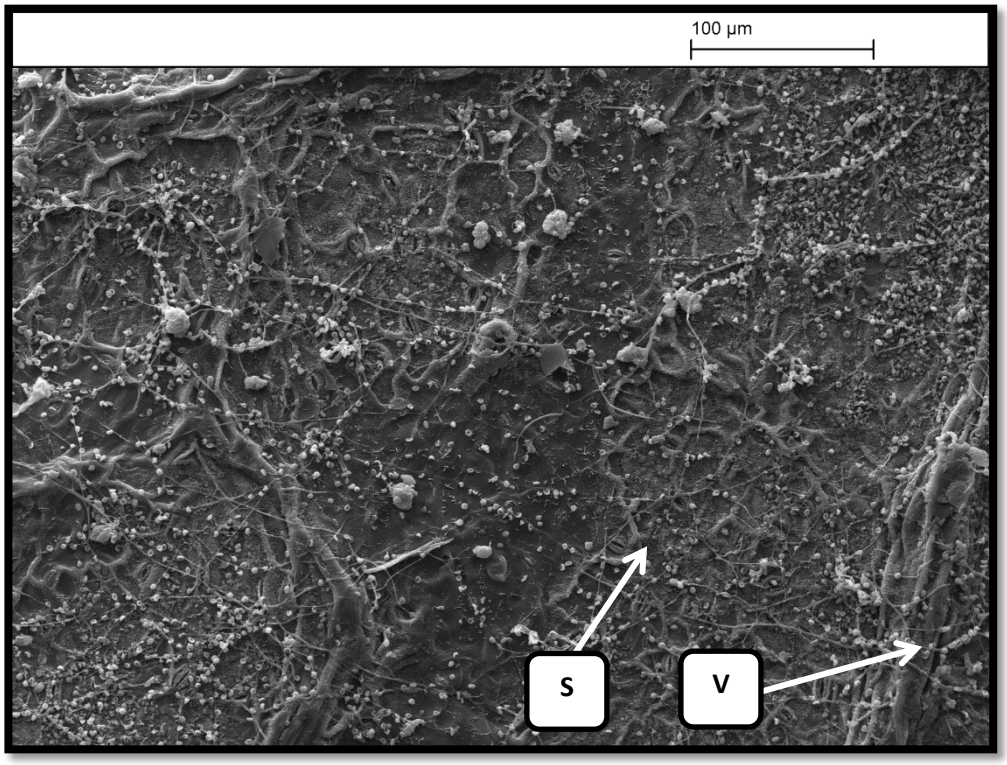

Figure 19: SEM image of a herbarium *S. cinerea* leaf collected from Pool Hall Pool (Staffordshire) in 1884; taken at 500x magnification, scale bar represents 100µm. Note that *S* is a stoma and *V* is a main vein.

An image of an epidermal scraping of Leaf 2 from Sample 17165a

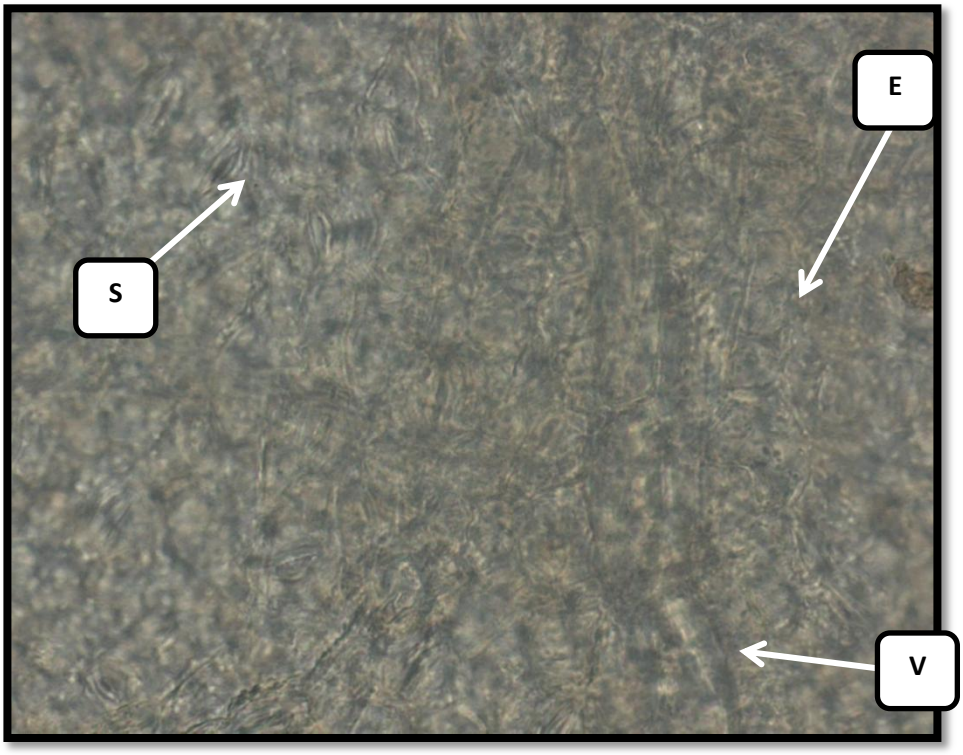

Figure 20: Epidermal scraping of a herbarium *S. cinerea* leaf; image taken at 400x magnification, using an Olympus compound microscope. Note that *S* is a stoma, *E* is an epidermal cell and *V* is a main vein.

Appendix 1 continued

An image of an epidermal scraping of a different area of the same leaf as shown in Figure 18

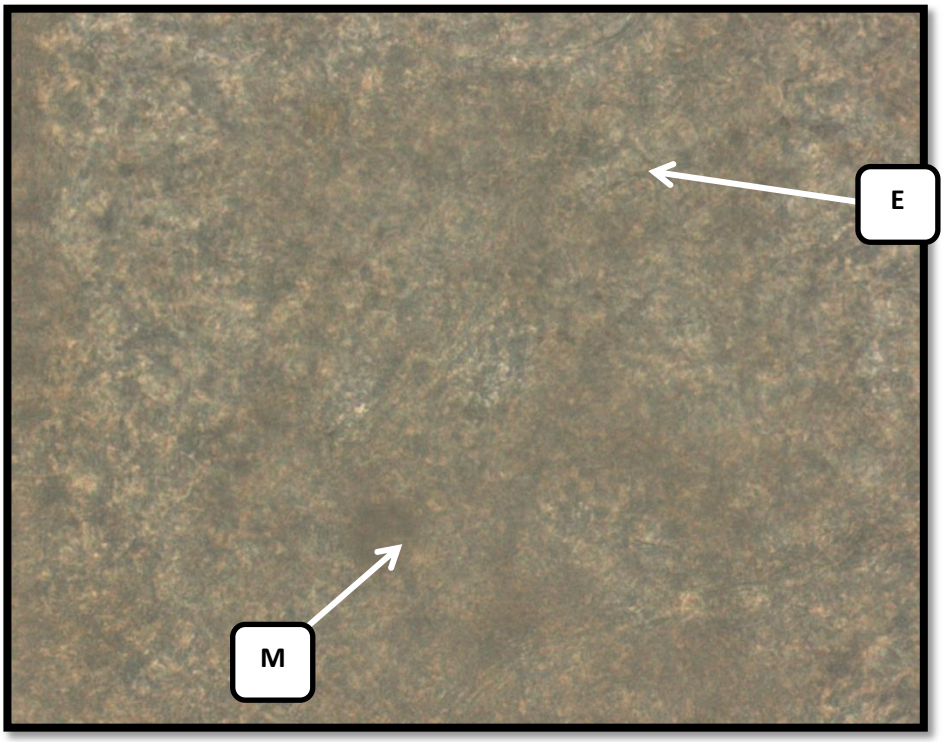

Figure 21: Epidermal scraping of a herbarium *S. cinerea* leaf; image taken at 400x magnification, using an Olympus compound microscope. Note that *E* is an epidermal cell and *M* is mesophyll tissue.

An image of the effect that the Chloroform wax removal method had on Leaf 6 from Sample SS4

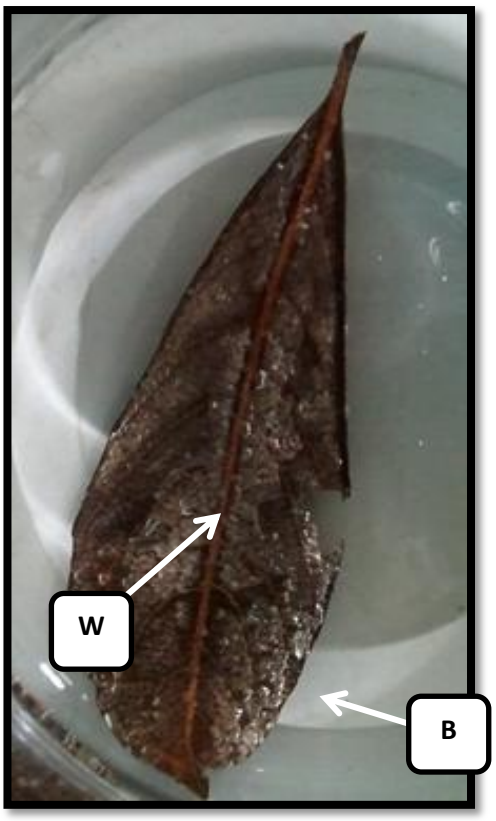

Figure 22: A photograph of the affect that the $CHCl_3$ wax removal method has on a herbarium *S. cinerea* leaf, collected from Pool Hall Pool (Staffordshire) in 1884. Note that *W* is partially smeared wax and *B* is a bubble of escaping gas.

An image of the effect that the Chloroform wax removal method had on Leaf 8 from Sample SS4

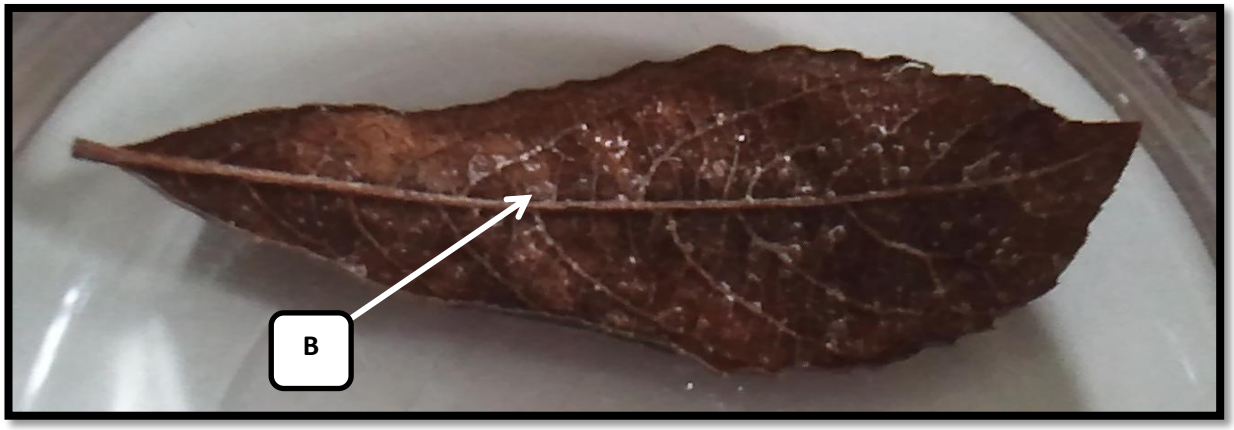

Figure 23: A photograph of the affect that the $CHCl_3$ wax removal method has on a herbarium *S. cinerea* leaf, collected from Pool Hall Pool (Staffordshire) in 1884. Note that *W* is partially smeared wax and *B* is a bubble of escaping gas.

Appendix 2

Table of SD Count Data

Sample Name/ Number	Collected From	Year	Mean Stomata (Edge)	Mean Stomata (Middle)	Mean Stomata (Overall)
17152	South Kilvington, Yorkshire	1854	313	406	360
17158	Trentham, Staffordshire	1873	146	73	110
17160	Pool Hall Pool, Staffordshire	1883	407	323	365
SS4 (17165a)	Pool Hall Pool, Staffordshire	1884	896	896	896
6861	Barmby Moor, Yorkshire	1931	94	146	120
6860	North Cave, Yorkshire	1955	365	396	381
B027	Mittlandsköggen, Sweden	2011	271	281	276
B112	Storesjö, Sweden	2011	104	167	136
B113	Storesjö, Sweden	2011	42	42	42
B217	Schwenningen Moos, Germany	2011	21	21	21
B309	Lord Brandon's Cottage, Ireland	2011	63	63	63
B310	Mangerton, Ireland	2011	417	375	396
B347	Muckross Peninsula, Ireland	2011	285	188	237
B445	La Ferme du Parc Jacques, France	2012	42	42	42
TH – L7	Trentham, Staffordshire	2012	208	156	132
TH – L8	Trentham, Staffordshire	2012	104	104	104
TH – L9	Trentham, Staffordshire	2012	21	104	63
TH – L10	Trentham, Staffordshire	2012	104	188	146
TH – L11	Trentham, Staffordshire	2012	188	313	251
TH – L12	Trentham, Staffordshire	2012	188	198	193
Pressed	Noddle Hill, Yorkshire	2012	31	31	31
Pressed	Noddle Hill, Yorkshire	2012	63	104	84
Pressed	Noddle Hill, Yorkshire	2012	63	125	94
Air Dried	Noddle Hill, Yorkshire	2012	21	21	21

Table 8: Mean stomatal counts from the edge, the middle and the whole leaf of samples examined via SEM.

Appendix 2 continued

Table of SD Count Data Continued

Sample Name/ Number	Collected From	Year	Mean Stomata (Edge)	Mean Stomata (Middle)	Mean Stomata (Overall)
Air Dried	Noddle Hill, Yorkshire	2012	63	21	42
Air Dried	Noddle Hill, Yorkshire	2012	208	146	177

Table 8 continued: Mean stomatal counts from the edge, the middle and the whole of the leaf of samples examined via SEM.

Appendix 3

Actual climate data used for SPSS analysis (see Section 5).

Site	Year	T (Mar)	TM (Mar)	Tm (Mar)	PP (Mar)	L (Mar)
Humberside	2012	8.30	13.48	3.48	0.00	12.11
Munich	2011	5.37	10.94	-0.23	0.00	12.12
Rennes	2012	9.65	16.15	3.29	1.02	12.16
Thorncliffe	2012	7.09	11.15	3.93	0.83	-
Valentia	2011	7.86	12.02	3.91	1.66	-
Växjö	2011	1.03	4.52	-2.68	0.00	12.03
Oxford	1854	6.70	11.20	2.20	10.60	-
Oxford	1873	5.40	9.70	2.20	60.10	-
Oxford	1883	1.90	7.20	-1.20	24.00	-
Oxford	1884	6.50	10.90	3.00	39.30	-
Sheffield	1931	4.20	7.30	-0.30	5.40	-
Sheffield	1955	3.20	6.50	-0.10	88.10	-
Stoke	-	-	-	-	-	12.12
Limerick	-	-	-	-	-	12.03

Table 9: Average climate data for March. Note that T = average temperature for the month (°C), TM = maximum average temperature for the month (°C), Tm = minimum average temperature for the month (°C), PP = average precipitation for the month (mm) and L = average amount of daylight (hours).

Site	Year	T (Apr)	TM (Apr)	Tm (Apr)	PP (Apr)	L (Apr)
Humberside	2012	7.72	11.20	4.13	0.00	14.29
Munich	2011	11.81	18.10	4.70	0.00	13.48
Rennes	2012	8.91	14.01	4.48	3.78	13.53
Thorncliffe	2012	5.23	8.87	2.49	4.76	-
Valentia	2011	11.72	15.59	8.07	2.18	-
Växjö	2011	9.32	15.07	2.73	0.00	14.10
Oxford	1854	9.20	15.00	3.50	19.90	-
Oxford	1873	7.70	12.80	3.90	12.20	-
Oxford	1883	8.10	14.00	4.50	25.70	-
Oxford	1884	7.20	11.70	3.10	40.20	-
Sheffield	1931	7.70	10.80	4.70	83.80	-
Sheffield	1955	9.30	13.50	5.90	19.00	-
Stoke	-	-	-	-	-	14.23
Limerick	-	-	-	-	-	14.14

Table 10: Average climate data for April. Note that T = average temperature for the month (°C), TM = maximum average temperature for the month (°C), Tm = minimum average temperature for the month (°C), PP = average precipitation for the month (mm) and L = average amount of daylight (hours).

Appendix 3 continued

Site	Year	T (May)	TM (May)	Tm (May)	PP (May)	L (May)
Humberside	2012	11.51	15.77	6.94	0.00	16.19
Munich	2011	14.54	20.58	7.77	0.00	15.38
Rennes	2012	13.97	19.61	8.58	2.13	15.00
Thorncliffe	2012	10.07	14.77	5.81	0.68	-
Valentia	2011	11.82	14.03	9.84	5.32	-
Växjö	2011	11.06	16.65	4.87	0.00	16.29
Oxford	1854	10.30	16.20	5.70	82.10	-
Oxford	1873	9.90	16.10	5.40	59.30	-
Oxford	1883	10.60	16.20	7.10	47.90	-
Oxford	1884	11.30	17.60	6.60	19.40	-
Sheffield	1931	11.40	15.30	7.20	78.30	-
Sheffield	1955	9.70	12.90	5.70	85.50	-
Stoke	-	-	-	-	-	15.10
Limerick	-	-	-	-	-	16.01

Table 11: Average climate data for May. Note that T = average temperature for the month (°C), TM = maximum average temperature for the month (°C), Tm = minimum average temperature for the month (°C), PP = average precipitation for the month (mm) and L = average amount of daylight (hours).

Site	Year	T	TM	Tm	PP	L	GS
Humberside	2012	9.18	13.48	4.85	0.00	14.20	-
Munich	2011	10.57	16.54	4.08	0.00	14.06	245
Rennes	2012	10.84	16.59	5.45	2.31	13.56	-
Thorncliffe	2012	7.46	11.60	4.08	2.09	-	-
Valentia	2011	10.47	13.88	7.27	3.05	-	332
Växjö	2011	7.14	12.08	1.64	0.00	14.14	295
Oxford	1854	9.31	14.13	3.80	37.53	-	308
Oxford	1873	8.98	12.87	3.83	43.87	-	271
Oxford	1883	9.02	12.47	3.47	32.53	-	285
Oxford	1884	9.83	13.40	4.23	32.97	-	315
Sheffield	1931	8.99	11.13	3.87	55.83	-	279
Sheffield	1955	9.28	10.97	3.83	64.20	-	299
Stoke	-	-	-	-	-	14.22	-
Limerick	-	-	-	-	-	14.06	-

Table 12: Average climate data for the spring months (March-May). Note that T = average temperature for the month (°C), TM = maximum average temperature for the month (°C), Tm = minimum average temperature for the month (°C), PP = average precipitation for the month (mm), L = average amount of daylight (hours), and GS = length of growing season (days).

Appendix 3 continued

Actual CO_2 data used for analysis in SPSS (see Section 5).

Site	Climate Data From	Year	CO_2
17152	Oxford/CET	1854	286
17158	Oxford/CET	1873	288
17160	Oxford/CET	1883	293
SS4	Oxford/CET	1884	294
6861	Sheffield/CET	1931	307
6860	Sheffield/CET	1955	316
B027	Växjö	2011	390
B217	Munich	2011	390
B309	Valentia	2011	390
B347	Valentia	2011	390
B445	Rennes	2012	392
Trentham	Thornecliffe	2012	392
Noddle Hill (Pressed)	Humberside	2012	392
Noddle Hill (Air Dried)	Humberside	2012	392

Table 13: CO_2 values to accompany the climatic data.

Appendix 4

Simple regression models using mean stomata density data as the constant and individual climatic factors as predictor variables (see Section 6.1.1 for analysis)

Overall Mean SD	B	SE B	β	Sig.
Constant (SD)	11.94	7.68		.138
Average Temperature of Spring Months (°C)	-0.66	0.88	-.174	.463
Note: R^2 = .030, ΔR^2 = -.024 (p = .463)				
Constant (SD)	20.59	9.55		.045
Average Maximum Temperature of Spring Months (°C)	-1.10	0.73	-.336	.147
Note: R^2 = .113, ΔR^2 = .064 (p = .147)				
Constant (SD)	7.21	3.81		.074
Average Minimum Temperature of Spring Months (°C)	-0.23	0.87	-.063	.791
Note: R^2 = .004, ΔR^2 = -.051 (p = .791)				
Constant (SD)	5.47	1.14		.000
Average Precipitation of Spring Months (mm)	0.18	0.11	.366	.113
Note: R^2 = .134, ΔR^2 = .086 (p = .113)				
Constant (SD)	5.11	1.32		.001
Length of Growing Season (Days)	0.01	0.01	.322	.166
Note: R^2 = .104, ΔR^2 = .054 (p = .166)				
Constant (SD)	-39.26	108.80		.722
Average Length of Daylight of Spring Months (Hours)	3.22	7.70	.098	.681
Note: R^2 = .010, ΔR^2 = -.045 (p = .681)				
Constant (SD)	-13.96	27.16		.614
Latitude	.00	.00	.173	.467
Note: R^2 = .030, ΔR^2 = -.024 (p = .467)				

Table 14: Results of simple regression analysis carried out in SPSS on mean stomatal density data as the response variable and climatic predictor variables individually. Where B is the unstandardized coefficient, SE B is the standard error of the B coefficient, β is the standardized coefficient and Sig. is the level of significance of the predictors' contribution to the model.

Appendix 4 continued

The contribution that each individual predictor variable makes to the mean stomatal density using numerical examples featured in the simple regression models of Table 14 (see Section 6.1.1 for analysis)

Overall Mean SD	Std. Deviation	β	Resulting Change
Constant (SD)	4.89		
Average Temperature of Spring Months	1.29	-.174	-0.85
Average Maximum Temperature of Spring Months	1.49	-.336	-1.64
Average Minimum Temperature of Spring Months	1.32	-.063	-0.31
Average Precipitation of Spring Months	9.90	.366	1.79
Length of Growing Season	149.70	.322	1.57
Average Length of Daylight of Spring Months (Hours)	0.15	.098	0.48
Latitude	22131.66	.173	0.85

Table 15: Contribution each predictor makes to the output (stomatal density), using numerical examples based on the results of simple regression analysis from Table 14. Where Std. deviation is the standard deviation, β is the standardized coefficient and Resulting Change is how much the outcome has altered due to each predictor (β multiplied by Constant's Standard Deviation).

The relationship between each predictor variable and the mean stomatal density constant, as well as the sample size (N) (see Section 6.1.1 for analysis)

Individual Predictors	Overall Mean SD	Sig.	N
Average Temperature of Spring Months	-.174	.231	20
Average Maximum Temperature of Spring Months	-.336	.074	20
Average Minimum Temperature of Spring Months	-.063	.395	20
Average Precipitation of Spring Months	.366	.056	20
Length of Growing Season	.322	.083	20
Average Length of Daylight of Spring Months (Hours)	.098	.340	20
Latitude	.173	.233	20

Table 16: The relationship between each climatic predictor and the output (stomatal density) using Pearson's Correlation Coefficient in the SPSS models has shown in Table 14.

Appendix 4 continued

Simple regression models (on modern English samples) using mean stomata density data as the constant and individual climatic factors as predictor variables (see Section 6.1.2 for analysis)

Overall Mean SD	**B**	**SE B**	**β**	**Sig.**
Constant (SD)	36.28	9.28		.008
Average Temperature of Spring Months (°C)	-3.82	1.11	-.815	.014
Note: R^2 = .664, ΔR^2 = .607 for Step 1 (p = .014)				
Constant (SD)	48.31	12.76		.009
Average Maximum Temperature of Spring Months (°C)	-3.49	1.02	-.815	.014
Note: R^2 = .664, ΔR^2 = .607 for Step 1 (p = .014)				
Constant (SD)	42.59	11.10		.009
Average Minimum Temperature of Spring Months (°C)	-8.52	2.48	-.815	.014
Note: R^2 = .664, ΔR^2 = .607 for Step 1 (p = .014)				
Constant (SD)	1.25	1.35		.390
Average Precipitation of Spring Months (mm)	3.14	0.91	.815	.014
Note: R^2 = .664, ΔR^2 = .607 for Step 1 (p = .014)				

Table 17: Results of simple regression analysis carried out in SPSS on mean stomatal density data (of modern English samples only) as the response variable and climatic predictor variables individually. Where B is the unstandardized coefficient, SE B is the standard error of the B coefficient, β is the standardized coefficient and Sig. is the level of significance of the predictors' contribution to the model.

Appendix 4 continued

The contribution that each individual predictor variable makes to the mean stomatal density using numerical examples featured in the simple regression models of Table 14 (see Section 6.1.2 for analysis)

Predictors	Std. Deviation	β	Resulting Change
Constant (SD)	4.31		
Average Temperature of Spring Months (°C)	0.92	-.815	-3.51
Average Maximum Temperature of Spring Months (°C)	1.00	-.815	-3.51
Average Minimum Temperature of Spring Months (°C)	0.41	-.815	-3.51
Average Precipitation of Spring Months (mm)	1.12	.815	3.51

Table 18: Contribution each predictor makes to the output (stomatal density) of modern English samples only, using numerical examples based on the results of simple regression analysis from Table 17. Where Std. deviation is the standard deviation, β is the standardized coefficient and Resulting Change is how much the outcome has altered due to each predictor (β multiplied by Constant's Standard Deviation).

The relationship between each predictor variable and the mean stomatal density constant, as well as the sample size (N) (see Section 6.1.2 for analysis)

Individual Predictors	Overall Mean SD	Sig.	N
Average Temperature of Spring Months	-.815	.007	8
Average Maximum Temperature of Spring Months	-.815	.000	8
Average Minimum Temperature of Spring Months	-.815	.000	8
Average Precipitation of Spring Months	.815	.000	8

Table 19: The relationship between each climatic predictor and the output (SD), using Pearson's Correlation Coefficient in the SPSS models has shown in Table 17.

Appendix 4 continued

A multiple linear regression model (on modern samples) using mean stomata density data as the constant and individual climatic factors as predictor variables (see Section 6.1.3 for analysis)

Predictors	B	SE B	β	Sig.
Constant (SD)	18.23	11.44		.133
Average Temperature of Spring Months (°C)	-4.19	6.53	-1.11	.531
Average Maximum Temperature of Spring Months (°C)	0.78	4.00	.236	.849
Average Minimum Temperature of Spring Months (°C)	2.75	2.80	.746	.343
Average Precipitation of Spring Months (mm)	0.20	0.21	.400	.357
Length of Growing Season (Days)	0.02	0.01	.500	.153
Note: R^2 = .373, ΔR^2 = .150 for Step 1 (p = .207)				

Table 20: Results of multiple regression analysis carried out in SPSS on mean stomatal density data as the response variable and climatic predictor variables. Where B is the unstandardized coefficient, SE B is the standard error of the B coefficient, β is the standardized coefficient and Sig. is the level of significance of the predictors' contribution to the model.

A multiple regression model (of all modern European samples) using mean stomata density data as the constant and climatic factors as predictor variables, including light (average length of daylight of the spring months) as a predictor (see Section 6.1.3 for analysis).

Predictors	B	SE B	β	Sig.
Constant (SD)	83.34	213.73		.703
Average Temperature of Spring Months (°C)	-2.50	8.74	-.658	.780
Average Maximum Temperature of Spring Months (°C)	-0.56	6.03	-.172	.927
Average Minimum Temperature of Spring Months (°C)	2.14	3.52	.580	.553
Average Precipitation of Spring Months (mm)	0.14	0.28	.293	.609
Length of Growing Season (Days)	0.02	0.01	.459	.235
Average Length of Daylight of Spring Months (Hours)	-4.20	13.77	-.128	.765
Note: R^2 = .378, ΔR^2 = .091 (p = .317)				

Table 21: Results of multiple regression analysis carried out in SPSS with mean stomatal density data as the response variable and climatic and light predictor variables. B is the unstandardized coefficient, SE B is the standard error of the B coefficient, β is the standardized coefficient and Sig. is the level of significance of the predictors' contribution to the model.

A multiple regression model (of all modern European samples) using mean stomata density data as the constant and climatic factors as predictor variables, including light (average length of daylight of the spring months) and latitude of sample site as a predictors (see Section 6.1.3 for analysis)

Predictors	B	SE B	β	Sig.
Constant (SD)	-812.33	979.72		.423
Average Temperature of Spring Months (°C)	-51.77	53.32	-13.658	.351
Average Maximum Temperature of Spring Months (°C)	36.23	39.73	11.042	.380
Average Minimum Temperature of Spring Months (°C)	22.99	22.53	6.224	.328
Average Precipitation of Spring Months (mm)	1.84	1.83	3.716	.335
Length of Growing Season (Days)	0.03	0.02	.951	.165
Average Length of Daylight of Spring Months (Hours)	29.89	38.93	.911	.457
Latitude	.00	.00	2.259	.367
Note: R^2 = .420, ΔR^2 = .082 for Step 1 (p = .353)				

Table 22: Results of multiple regression analysis carried out in SPSS on mean stomatal density data as the response variable and climatic, light and latitude predictor variables. B is the unstandardized coefficient, SE B is the standard error of the B coefficient, β is the standardized coefficient and Sig. is the level of significance of the predictors' contribution to the model.

A multiple regression model (of modern English samples only) using mean stomata density data as the constant and climatic factors as predictor variables, excluding light and latitude of the sample location as predictors because they are not necessary with samples of such a close distribution. It should be noted that most variables have been presented as 'excluded' as they did not meet the criterion for SPSS to include them in, thus rendering the mode now a standard single regression model (see Section 6.1.3 for analysis)

Overall Mean SD	**B**	**SE B**	**β**	**Sig.**
Constant (SD)	1.25	1.35		.390
Average Temperature of Spring Months (°C)	Excluded	Excluded	Excluded	Excluded
Average Maximum Temperature of Spring Months (°C)	Excluded	Excluded	Excluded	Excluded
Average Minimum Temperature of Spring Months (°C)	Excluded	Excluded	Excluded	Excluded
Average Precipitation of Spring Months (mm)	3.14	0.91	.815	.014
Note: R^2 = .664, ΔR^2 = .607 for Step 1 (p = .014)				

Table 23: Results of multiple regression analysis carried out in SPSS on mean stomatal density data as the response variable and climatic predictor variables (of modern English samples only). B is the unstandardized coefficient, SE B is the standard error of the B coefficient, β is the standardized coefficient and Sig. is the level of significance of the predictors' contribution to the model.

A simple regression model using mean stomata density data as the constant and CO_2 as the predictor variable (see Section 6.2 for analysis)

Predictors	**B**	**SE B**	**β**	**Sig.**
Constant (SD)	11.97	10.72		.279
Carbon Dioxide Levels (ppmv)	-0.02	0.03	-.14	.547
Note: R^2 = .020, ΔR^2 = -.034 for Step 1 (p = .547)				

Table 24: Results of single linear regression analysis carried out in SPSS on mean stomatal density data as the response variable and CO_2 data as the predictor variable (for English samples). Where B is the unstandardized coefficient, SE B is the standard error of the B coefficient, β is the standardized coefficient and Sig. is the level of significance of the predictor's contribution to the model.

A multiple regression model (of English samples) using mean stomata density data as the constant and CO_2 and climatic factors as predictor variables (see Section 6.3 for analysis)

Predictors	B	SE B	β	Sig.
Constant (SD)	-2.28	7.68		.771
Average Temperature of Spring Months (°C)	23.63	9.30	15.508	.025
Average Maximum Temperature of Spring Months (°C)	4.95	5.34	4.687	.371
Average Minimum Temperature of Spring Months (°C)	-55.88	28.81	-17.419	.074
Average Precipitation of Spring Months (mm)	0.68	0.66	2.236	.325
Length of Growing Season (Days)	-0.29	0.19	-5.831	.144
Carbon Dioxide Levels (ppmv)	0.01	0.02	.051	.771
Note: R^2 = .831, ΔR^2 = .753 for Step 1 (p < .001)				

Table 25: Results of multiple regression analysis carried out in SPSS on mean stomatal density data as the response variable and CO_2 and climate data as the predictor variables (of English samples). Where B is the unstandardized coefficient, SE B is the standard error of the B coefficient, β is the standardized coefficient and Sig. is the level of significance of the predictors' contribution to the model.

A multiple regression model (of all modern European samples) using mean stomata density data as the constant and CO_2 and climatic factors as predictor variables, including light (average length of daylight of the spring months) as a predictor (see Section 6.3 for analysis)

Predictors	B	SE B	β	Sig.
Constant (SD)	975.85	664.98		.166
Average Temperature of Spring Months (°C)	-1.57	8.32	-.414	.853
Average Maximum Temperature of Spring Months (°C)	-1.36	5.71	-.414	.816
Average Minimum Temperature of Spring Months (°C)	1.93	3.41	.523	.580
Average Precipitation of Spring Months (mm)	0.13	0.27	.263	.641
Average Length of Daylight of Spring Months (Hours)	-5.91	13.32	-.180	.664
Carbon Dioxide Levels (ppmv)	-2.21	1.77	-.442	.235
Note: R^2 = .378, ΔR^2 = .091 for Step 1 (p = .317)				

Table 26: Results of multiple regression analysis carried out in SPSS on mean SD data in relation to climate and CO_2 data of all European samples. Where B is the unstandardized coefficient, SE B is the standard error of the B coefficient, β is the standardized coefficient and Sig. is the level of significance of the predictors' contribution to the model.

Appendix 4 continued

The contribution that each predictor variable makes to the mean stomatal density using numerical examples featured in the multiple regression model of Table 24 (see Section 6.3 for analysis)

Predictors	Std. Deviation	β	Resulting Change
Constant (SD)	6.46		
Average Temperature of Spring Months (°C)	4.24	15.508	100.18
Average Maximum Temperature of Spring Months (°C)	6.12	4.687	30.28
Average Minimum Temperature of Spring Months (°C)	2.01	-17.419	-112.53
Average Precipitation of Spring Months (mm)	21.32	2.236	14.44
Length of Growing Season (Days)	128.31	-5.831	-37.67
Carbon Dioxide Levels (ppmv)	48.95	.051	0.33

Table 27: Contribution each predictor makes to the output (stomatal density) in English samples, using numerical examples based on the results of multiple regression analysis from Table 24. Where Std. deviation is the standard deviation, β is the standardized coefficient and Resulting Change is how much the outcome has altered due to each predictor (β multiplied by Constant's Standard Deviation).

The contribution that each predictor variable makes to the mean stomatal density using numerical examples featured in the multiple regression model of Table 25 (see Section 6.3 for analysis)

Predictors	Std. Deviation	β	Resulting Change
Constant (SD)	4.89		
Average Temperature of Spring Months (°C)	1.29	-.414	-2.02
Average Maximum Temperature of Spring Months (°C)	1.49	-.414	-2.02
Average Minimum Temperature of Spring Months (°C)	1.32	.523	2.56
Average Precipitation of Spring Months (mm)	9.90	.263	1.29
Average Length of Daylight of Spring Months (Hours)	0.15	-.180	-0.88
Carbon Dioxide Levels (ppmv)	0.98	-.442	-2.16

Table 28: Contribution each predictor makes to the output (stomatal density) in all European samples, using numerical examples based on the results of multiple regression analysis from Table 25. Where Std. deviation is the standard deviation, β is the standardized coefficient and Resulting Change is how much the outcome has altered due to each predictor (β multiplied by Constant's Standard Deviation).