Behavioural Characters in Phylogenetics: A Case Study
Using Black Fly (Diptera: Simuliidae) Cocoon Spinning
Behaviour

by

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ABSTRACT

Historically, the predominant method of reconstructing phylogenies has been through the use of morphological characters. There are new techniques now gaining acceptance, including molecular techniques and chromosomal information.

Although the study of behaviour has been used in a comparative framework, these analyses have, historically, been based on intuition. Hennig (1966) devised a new method of reconstructing phylogenies which provided a noncircular method for formulating, testing and refining phylogenies. Subsequent systematists had virtually abandoned ecological and behavioural data as primary indicators of phylogenetic relationships (Brooks and McLennan 1991). Therefore, in a modern cladistic framework (sensu Hennig) the analysis of behavioural traits remains underrepresented as a method of reconstructing phylogenies. This thesis will reconstruct the phylogeny for species of black flies (Diptera: Simuliidae), using two steps. The first step is to thoroughly understand and explain the cocoon spinning in black fly larvae. There have been 5 previous descriptions of cocoon spinning, but all were incomplete or erroneous. The advances in technology, including video recorders and VCRs, have allowed this behaviour to be analyzed in great detail in 20 different species. A complete description of the cocoon spinning of Simulium vittatum is given. This description will be used as a template for the other species observed.

The description and understanding of cocoon spinning was the first step in undertaking a phylogenetic analysis using this behaviour. The behaviour was then broken down and analyzed, revealing 23 characters,
either qualitative and quantitative in nature. These characters were assessed in a cladistic framework (sensu Hennig) and a phylogenetic tree was reconstructed with a C.I of 0.91 and an R.I. of 0.96. This phylogenetic tree closely resembles a previously established phylogenetic tree produced from morphological and cytological information.

The importance of this result is the indication that, contrary to some authors, behavioural characters, if used properly, can add very informative characters to a data set.
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1.0 INTRODUCTION

Black flies are the perfect organisms for a project combining behaviour and phylogenetics because they possess a complex behaviour which can be analyzed quite easily. The black fly spins a cocoon where it resides as a pupa. The cocoon spinning behaviour is complex, and when thoroughly described will yield a number of characters to be used in a phylogenetic analysis. The fact that black flies are well studied, due to their economic and health significance, has led to a very good understanding of relationships among black fly species. This study therefore, is a combination of producing a behavioural phylogeny, and comparing this to existing phylogenies to assess the validity of using behavioural characters in a phylogenetic context.

1.1 BLACK FLY LIFE HISTORY

Black flies pass through 4 stages to complete their life history: the egg, larva, pupa and adult. All black fly larvae are aquatic. They attach to various submerged objects, including sticks, rocks, grasses and leaves. They are found in many different types of lotic environments ranging from large rivers to tiny spring-fed trickles and from swift currents to almost stagnant water. Larvae of some species are only able to exist in one of these types of environments, whereas others can be found under many different environmental conditions.

After eclosion, larvae often remain at the site of hatching if suitable attachment sites are available and food supply is adequate. If the area is not suitable the larva will float downstream connected by a strand of silk to another location (Peterson 1981). Larvae pass
through 4-9 larval molts, depending on the species, with 7 being most common.

The last instar larvae (pharate pupae) feed and spin cocoons of varying constant shapes depending on the species that serve to protect and anchor the developing pupae (Hinton 1958). In the pharate pupa the pupal cuticle is fully formed beneath the old larval cuticle. The two cuticles have already parted (apolysis) and the pharate pupa's muscles, attached via apodemes to the larval structures, are used to move the old larval structures. Morphological transformation into the stage which is commonly referred to as the pupa does not occur until the old larval coat is shed (ecdysis) (Hinton 1958). However, even though it is the pharate pupa that spins the cocoon, it still outwardly resembles a larva, and therefore, from this point on, the term larva will be used.

At the completion of the cocoon spinning process, the pupa breaks out of the larval skin and wriggles down into the cocoon that has been built. The black fly now has the appearance of a typical pupa. While inside the cocoon, there are hooks on the dorsal surface of the pupa to anchor the individual to the roof of the cocoon. The pupal stage lasts 4-7 days, but varies with water temperature (Peterson 1981). During this time the pupa relies on the respiratory filaments for gas exchange. These protrude out of the mouth of the cocoon. During the pupal stage the black fly does not consume any food or move about, it is anchored in the location where the cocoon was built.

The emerging adult breaks and pulls itself out of the pupal skin. Its wings expand and the adult rises to the surface of the water in an
air bubble. Once at the surface the adult climbs to a nearby support to rest and allow its cuticle to harden (Peterson 1981).

After emergence, the female will mate (excluding parthenogenetic species). In many species the female then requires a blood meal in order for the eggs to mature (anautogeny). A female will produce 200-500 eggs in a single gonotrophic cycle and most species will oviposit in or at the edge of running water; however, some species can oviposit in almost still water. Depending on the species, oviposition can be performed on the surface of the water, on substrates that are sprayed with vigorous currents or even underwater (Golini and Davies 1987).

Incubation of the eggs takes between 4-30 days unless the eggs pass through diapause, in which case, it can take much longer. There is an egg burster on the head of the first instar larva to assist in breaking out of the egg, thus completing the life cycle (Peterson 1981).

1.2 ECONOMIC AND HEALTH SIGNIFICANCE

Black flies are serious pests to humans and other homiothermic animals in many parts of the world and may cause them enormous distress. Black fly females feed on humans, birds or other animals but some do not blood feed at all (autogenous species). Their bite can produce severe initial irritation often accompanied by toxic and allergic reactions (Kim and Merritt 1987). Therefore, all black flies that feed on blood can be considered pests.

There are two main economic costs associated with black flies. One is the costs of loss of production in livestock and poultry farming. The bites themselves have been responsible for illness and mortality in various wild and domestic animals. Therefore, there are economic
losses in production and control costs for such agricultural industries as poultry, beef and dairy cattle farming (Kim and Merritt 1987).

The second major economic cost is related to disease in humans. There are a number of diseases that are vectored by different black fly species, and this is of social as well as economic importance. The public health risk is greatest where black flies serve as vectors of the filarial nematode *Onchocerca volvulus* which causes human onchocerciasis (river blindness) in Tropical Africa, Central America, northern South America and Yemen. There are about 18 million people infected around the world resulting in severe visual impairment or blindness for 2 million (Berger and Nnadozic 1993).

Black flies are also vectors of other organisms, which affect animals. *Simulium venustum* is known to vector the nematode *Difoliaria ursi* to black bears (Addison 1980). Some black flies are known to transmit various avian blood protozoans including *Trypanosoma* spp. and several species of *Leucocytozoon* (Fallis et al. 1974). *Simulium vittatum* has been shown to be a vector of the New Jersey serotype of vesicular stomatitis virus (Cupp et al. 1992). This is a disease of livestock (horses, cattle and swine), certain wildlife species (ungulates and swine) and humans (Webb and Holbrook 1989). Black flies of an unnamed *Simulium* sp. are possible vectors of blue-tongue and epizootic hemorrhagic disease virus transmission to wildlife species, such as the bighorn sheep (*Ovis canadensis cremnobates*) (Mullens and Dada 1992).

A third economic cost may be the loss of income from decreased tourism, but this is not well documented.

**1.3 CLASSIFICATION AND PHYLOGENETICS**
Biological entities have long been named and described, and attempts have been made to place all organisms in groups which represent the course of evolution. There are major differences, however, between the description and identification of a species and understanding the evolutionary relationships among species. The former is taxonomy and identification and the latter, phylogenetics. Identification is the use of characters to place an individual into a species grouping, and taxonomy is the naming of the groupings, whether that be species, or some higher taxonomic level. In taxonomy organisms are arranged, but only into a convenient classificatory system (Quicke 1993).

The characters that are used to identify a species are not necessarily the same ones that are used to reconstruct a phylogeny. This is because the characters that separate a species from all the other species tend to be autapomorphic (unique derived) characters. Only characters that are synapomorphic (shared derived) can be used in a cladistic analysis among species (Hennig 1966).

The earliest documented attempt to understand the evolution of animal species was Aristotle who divided animals into two main divisions, animals that have blood and those that do not have blood. Lamarck (1914) believed that this was false, though, because he felt that the direction of the evolution was backwards, from most complex (with blood) to most simple (without blood). Lamarck thus divided the animal kingdom into two different groups, animals that have vertebrae and those without vertebrae (Lamarck 1914).

The groupings describe one category which has the presence of a character which is useful phylogenetically because it is a shared derived character. The other category, however, lumps together everything that lacks the character, and thus is not useful in a phylogenetic context because the grouping is based on sympleisotypies (shared ancestral traits). Despite the obvious flaws of 'A' and 'not A' groupings, they remain prevalent in the classification of organisms.

In the study of evolution, and the attempt to classify organisms with most closely related species, there has been a progression in methodology. There have been three methodologies employed, now referred to as: evolutionary systematics, endorsed by Mayr and Simpson, numerical taxonomy or phenetics, originated by Sneath and Sokal (1973) and phylogenetic systematics or cladistics as described by Hennig (1965) (Minelli 1993).

The first system is now deemed the evolutionary systematic or taxonomic system. This method employs intuition as the method of classifying organisms and grouping them. Characteristics that are used to produce groupings are selected only by the intuition of the author. Any that are deemed important are used, and their usefulness will outweigh other characteristics, which are not believed to be as important evolutionarily. If there are differences in opinion on which characteristics are important or how important any one is relative to others, inconsistencies will arise. It is almost impossible to be sure whether groups classified by intuition are significant evolutionarily because they may not represent anything real in nature, and the grouping may be artificial (Wiley et al. 1991). Many comparative
studies continue to use this system as their baseline, even though there are systems in place (i.e. phylogenetics) which more accurately represent evolution (Harvey and Pagel 1991).

The phenetics system is an empirical method for determining taxonomic relationships. It was largely developed and popularized by Sneath and Sokal (1973). Pheneticists believe that relationships should be based on overall similarity. Therefore, they include all characters and give them all equal weight. The trees produced are considered to be unbiased indicators of similarity or difference between the taxa because all the characters are included (Quicke 1993).

The phenetics system is slightly better than the evolutionary taxonomic system because it does select characters and uses a data matrix, to allow for reproduction of the tree produced. The problem with this method is that the algorithms concentrate on reflecting the total similarity of the organisms. This allows similar organisms to be grouped together ignoring parallelisms and convergences and therefore the groupings may be artificial (Wiley et al. 1991; Harvey and Pagel 1991). The pheneticists believe that the inclusion of any evolutionary knowledge, which would reject certain characters, makes the process biased. However, the a priori selection and weighting of characters is not only desirable in phylogenetics, it is necessary (Hull 1994).

The cladistics system which was described by Hennig (1965, 1966) is now the system of choice for determining the pattern of relationships among taxa. The basis of this system is the use of shared derived characters (synapomorphies) to determine relationships.
Hennig (1965, 1966) proposed a concept where the relationships among species is crucial for phylogenetics. He describes the "phylogenetic relationship" as a concept where species B is more closely related to species C than to another species, A, when B has at least one ancestral species source in common with C which is not the ancestral source of species A.

Table 1 is a hypothetical data matrix, indicating how the different systems can give different phylogenies. The phenetics system would have taxa A and B most closely related with taxon C as their sister group, because A and B share the most characters in common (Figure 1, A). In the cladistic system 0 is considered primitive and 1 derived. Therefore, this system would have taxa B and C most closely related with taxon A as their sister group, because only character 2 is a shared derived character and it is shared by taxa B and C (Figure 1, B). In a cladistic system, none of the other characters are used because they are all autapotypies (unique derived character). Character 1 is unique to taxon B, and characters 3, 4 and 5 are unique to taxon C.

It is difficult to hypothesize the relationships using the traditional system, because it would depend solely on which of these characters was believed, by the taxonomist, to be the most important. If only the 'characters' in Table 1 were used, the tree would likely resemble the phenetic tree because taxa A and B would likely look most similar.

Hennig (1965, 1966) introduces other important concepts. One is the monophyletic group and another is the principle of parsimony,
Table 1: Hypothetical data matrix for three taxa and five characters.

Figure 1: Phylogenetic trees produced using the matrix in Table 1 under A) the phenetic system and B) the cladistic system.
Character 1 2 3 4 5

- taxa A: 0 0 0 0 0
- taxa B: 1 1 0 0 0
- taxa C: 0 1 1 1 1
which are both fundamental to an understanding of cladistics. The
monophyletic group is a grouping, whether small or large, whose
members are more closely related to one another than to species
which stand outside the group.

It is highly unlikely that all the characters used, will fit to make
one single tree, mostly because convergence and parallelisms do occur
in nature, and this is where the principle of parsimony must be
employed. This principle states that the most likely phylogenetic
explanation must be the one requiring the least number of
evolutionary steps (including reversals, convergences or parallelisms)
(Farris 1982).

It is clear that there are problems associated with these three
methods of phylogenetic analysis. There are two main criticism of
cladistics. One is that the system does not take into account factors
such as: the degree of divergence or the amount of diversification
(patristic affinity) (Hull 1994). While the other is that the
phylogenetic information is retained as the sole criterion for
reorganizing and ranking taxa (Mayr 1994). Some also believe that
the concepts of the monophyletic group and the principle of
cparsimony are not fundamentally necessary for classification which
represents evolution (see Farris 1994).

The phenetic system does not consider evolution in the analysis
and therefore accepts convergent characters. Thus, it is difficult to
reconstruct an appropriate phylogeny that represents evolution
(Wiley et al. 1991; Harvey and Pagel 1991). The remainder of
biologists, who believe that evolution must be considered in
phylogenetic analyses, are faced with a dilemma. On one side there is
Hennig's system, "where cladistic development is inferable from a classification, but lost is the information about patristic affinities and the cumbersome classifications that would result" (Hull 1994). On the other side there is evolutionary taxonomy where one would have to abandon the ideal that classifications imply anything very precise about the phylogenetic development (Hull 1994). It appears, therefore that for phylogenetic analyses or classification, that presently cladistics is the best system available.

The goal of this thesis is to most accurately reconstruct the phylogeny of the black flies using the cocoon spinning as the characters for the phylogenetic analysis. It is not imperative to determine the degree of divergence, the amount of diversification, or to reorganize or rank the species of black flies; therefore, the system proposed by Hennig (1966) will be used in this thesis.

2.0 LITERATURE REVIEW

2.1 CLASSIFICATION

There are approximately 1600 named valid species of black flies worldwide (Dr. R. Crosskey, Natural History Museum, London, pers. comm., 1995). The family Simuliidae is very homogeneous structurally and easily recognized. This causes some taxonomic problems because it reduces the number of distinctive morphological characters that can be used to differentiate among taxa (Peterson 1981).

Most of the taxonomy has been done using morphological characters of both the adult and immature stages (excluding the egg stage). However, there are a number of other methods, usually used in combination with morphology, of classifying a species, including
cytotaxonomy, some fossil records and chemotaxonomy (including molecular work) (Crosskey 1987).

2.1.1 Morphotaxonomy

The use of morphological characters remains the dominant method of classification in the simuliids. There is scarcely a morphological attribute in any life stage or in either sex that has not been well studied. Some morphological features are used as characters (i.e., traits that have a use taxonomically) while others have been scrutinized mainly to determine their probable functions (Crosskey 1987).

In the larval stage some of the more common morphological characters used in species diagnosis are the labrum, antenna, hypostomium, larval headspot pattern, proleg and abdomen (Wood et al. 1963; Currie 1986). In the pupal stage the structure of the cocoon and the number and form of the respiratory filaments are predominantly used for identification (Stone 1964). The adult stage is where most of the identifications are positively made to the species level, using morphological details of the head, thorax, wings, legs, abdomen and genitalia (Davies et al. 1962).

2.1.2 Cytotaxonomy

It has been discovered that most widespread, common black fly morphospecies are actually sibling species complexes. These are reproductively isolated but morphologically similar populations within a previously established morphospecies (Rothfels 1979).

Most sibling species have been revealed using cytological information (Adler 1987). However, less than 10% of the world total
of named morphospecies has been studied chromosomally (Crosskey 1987).

Typically the polytene chromosomes of the larval salivary glands are studied (Rothfels and Dunbar 1953), although some progress has been made using the polytenes of adult Malpighian tubules (insect kidneys) (Procunier and Post 1986).

Sibling species can be described in terms of gross features of the salivary gland chromosomes, including the length of chromosome arms, position and morphology of centromere regions, presence or absence of a chromocenter, and the positions of the nucleolar organizer, Ring of Balbiani and other major puffs (Rothfels 1981). However, the analysis of the chromosomal banding patterns is the predominant tool of cytotaxonomy. Differences in chromosome banding pattern allow for the recognition of biologically distinct sibling species among larvae that were considered isomorphic (Rothfels 1979). These bands can reveal fixed rearrangements, differences in the kind and frequency of floating inversions and specific features of the sex chromosomes (Rothfels 1981). Inversions are the result of two breaks in the chromosome and therefore the probability of an identical inversion occurring independently more than once is very small (Rothfels 1979). Therefore, individuals sharing the same banding pattern for a given stretch of chromosome are considered to do so by virtue of common descent.

Very little work has been done on adult Malphigian tubule chromosomes. The advantage of using adult chromosomes is that the same chromosomal characters can be used for the adults as used originally to describe each of the sibling species (i.e. in the larval
stage). This is because the polytene chromosome banding pattern is conserved between adult and larval tissues (Procunier and Post 1986). Also, after a black fly has taken a blood meal from its host (human or livestock), it is important to identify the species in order to determine whether it was a vector of a harmful organism (Procunier and Post 1986).

2.1.3 Chemotaxonomy

This is becoming a newly explored area of black fly taxonomy. There are a few techniques that have not proven to be reliable yet, and also some new techniques that appear to have potential for both identifying species as well as for use in phylogenetic reconstruction. One of the latest techniques is cuticular hydrocarbon analysis by gas liquid chromatography. This technique uses the cuticular wax, because it has been shown that closely related species vary in their cuticle hydrocarbons (Townson et al. 1987). The advantages of this technique are that it does not require specimen destruction and it can be used on an isolated cuticular structure such as a single wing (Crosskey 1987).

Enzyme electrophoresis is another technique used, but it has been found to be insufficient for identifying sibling species (Townson et al. 1987). DNA-DNA hybridization has not reached its full potential in simuliid taxonomy largely because formally it was a slow, laborious process that required a large amount of DNA (Townson et al. 1987).

Advances in DNA technology now allow types of molecular techniques, that previously were only theoretical, to become extremely useful for identification and phylogenetics. Brockhouse et al. (1993) described two molecular assays to differentiate among
sibling species. One relies on polymerase chain reaction (PCR)-mediated amplification of internal transcribed spacers of the nuclear rDNA loci. The second technique involves immunoblotting of silk proteins that are found in larvae and adult black flies. Brockhouse et al. (1993) believe that a combination of both of these techniques will allow for unambiguous identification.

DNA sequences vary in three ways: 1) the chromosomal location of a sequence can be altered, 2) the copy number of repetitive sequences can be changed by deletion or amplification and 3) the exact base sequence can be altered. All these types of sequence changes can indicate differences among species (Post and Crampton 1988).

2.2 PHYLOGENETICS

2.2.1 Phylogenetics of Black Flies

All the taxonomic methods mentioned above have phylogenetic potential. The characters will be different than those used to identify species, but the methodology to attain the characters can be the same. For example, there are many morphological characters that can be used in a cladistic framework. However, these characters are often not be the same as the morphological characters used for taxonomy (see section 1.3).

The black fly phylogenies in existence today are based predominantly on morphological characters (Crosskey 1987). However, systematists are now interested in the phylogenetic potential of molecular data to expand the morphology based phylogenies. The combination of all the taxonomic methods (used to attain phylogenetic characters) mentioned previously is the most
successful way of constructing phylogenies that truly represent evolution.

When chromosomal analyses are extended to a sufficient number of species a chromosomal phylogeny can be constructed based on shared rearrangements, principally inversions (Rothfels 1981). Cytophylogenies have been shown to be largely congruent with morphophylogenies when established independently (Rothfels 1981).

As a technique for phylogenetic analysis it is now possible to use the variation in the mitochondrial DNA (mtDNA) sequence of various black fly species. This involves using PCR to amplify mtDNA \textit{in vitro}. Primers that are derived from nucleotide sequences of one species can be used to amplify homologous regions in closely or distantly related species. It appears that mtDNA sequences may reveal enough detail for phylogenetic analysis as well as aiding in the taxonomic resolution of species complexes (Xiong and Kocher 1991).

2.2.2 Behavioural Phylogenetics

Behavioural information has been used for identification of animal species for quite some time. In a comparative framework, Niko Tinbergen, Konrad Lorenz and other workers (see Brooks and McLennan 1991) did considerable work on behaviour and also attempted to understand the relationships among the animal species they studied (Tinbergen 1951). However, the use of behaviour in phylogenetics \textit{sensu} Hennig, has been quite limited (Brooks and McLennan 1991). There has also been some debate in the past over the validity of the use of behavioural characters in cladistic analyses because they were said to be more labile than morphological characters, and that convergence is more common due to the
pressures of natural selection on behaviour (DeQueiroz and Wimberger 1993). Atz (1970) believes that homology is a concept that relates to structural correspondence of characters and is only meaningful in behaviour to the extent that behaviour can be related to structure. He believes, though, that if behaviours are analyzed as if they are structure, then behaviour has little or no purpose as a phylogenetic tool (Atz 1970). These apprehensions have persisted, and a vast data base of ecological and behavioural characters remains virtually unexplored by systematists (Brooks and McLennan 1991).

There have been accounts of analyses using both behavioural and morphological characters where the authors indicate that the behavioural information is not as useful phylogenetically (Carpenter 1987; Urbani 1989). Carpenter (1987) studied 32 morphological and 7 behavioural characters in wasps (Hymenoptera: Vespidae). He determined that the behavioural data were ambiguous relative to the morphological data. Urbani (1989) produced a phylogeny of ants based on 26 morphological characters and one behavioural character. He describes the evolution of a number of other behavioural characters in his paper, which were mapped onto the phylogeny produced. Urbani (1989) states that it seems difficult to use purely behavioural traits to characterize major evolutionary trends, although he does admit that subdividing the complex behaviours (which he was analyzing) into simpler components might provide good characters useful for a phylogenetic analysis.

However, there are studies which contradict the assumption that behaviour is more evolutionarily plastic. In these studies, behavioural characters have been used in phylogeny reconstruction. McLennan et
al. (1988), working on stickleback fishes, used the behaviour of mating rituals as characters for phylogeny reconstruction of the group. According to these authors, behaviour is closely associated with physiology, development and morphology and is subject to the same evolutionary processes and constraints, therefore it should change in ways that reflect underlying phylogenetic relationships. McLennan et al. (1988) state that behaviour is rarely used because it is dynamic and therefore potentially difficult and costly to study, however, it is not impossible.

Prum (1990) performed three analyses of Manakin (Aves: Pipridae) display behaviour to determine whether behaviour could be useful in a phylogenetic context. In all analyses 44 behavioural characters were used. The analyses were 1) using the behavioural characters and comparing the phylogeny produced with a morphological phylogeny, 2) using both behavioural and morphological information to reconstruct a phylogeny and 3) mapping the behavioural characters onto the existing morphological phylogeny. The results confirmed the applicability of using behavioural information in a phylogenetic analysis.

Arntzen and Sparreboom (1989) studied Old World newts and found that when using protein electrophoretic data (19 characters) and behavioural data (16 characters, all relating to sexual behaviour—predominantly male courtship) a single fully resolved and robust phylogeny was produced. This particular behaviour shows a number of shared elements, which Arntzen and Sparreboom (1989) feel make this behaviour more suitable for phylogenetic inferences than other types of behaviour.
McLennan et al. (1988) indicated that behavioural homoplasy is no more prevalent than morphological homoplasy. In some cases, behavioural data appear to be more stable than morphological data and show lower average homoplasy across the same taxa (Wenzel 1992). If several species show the same complex movements in the same context and the behaviour appears to be largely innate they may be thought of as homologous. The postulate of homology will be stronger, the more complicated and distinctive the behaviour (Wenzel 1992).

All authors seem to agree, however, that behaviour as used for phylogenetic reconstruction has been most useful to date, in low level taxonomic studies (i.e., genus and species) (Atz 1970, Hodos 1976, McLennan 1988). This is due to the difficulty in finding all the organisms in the natural environment to study. The principle difficulty in comparing behavioural patterns at a higher level (i.e., family or order) is the identification of a similar behavioural pattern in a number of different families. Therefore the evaluation of their status as distinct characters and subsequently, the determination of homology is difficult (Arntzen and Sparreboom 1989). However, this does not imply that behaviour is more homoplasious than morphology.

Tinbergen (1959), (pre-Hennig) stated that “behavioural characters are in principle neither more nor less useful than morphological or other characters, they merely add characters to the total by which overall likeness is judged.”

2.2.3 The Behaviour Of Building an External Structure

There are a variety of insect groups that build some sort of architectural structure. Whether these are cocoons, webs or nests, the
behaviour of construction has been studied in a number of groups. One of the earliest studies was performed by Emerson (1938), who studied termite nests. The nest structures are morphological expressions of behavioural patterns. This quality makes aspects of behavioural evolution as visible as morphological evolution. These nests are built by a number of individuals, but the pattern of the nest is species specific (Emerson 1938). It is important to understand that although the end products of a particular behaviour may be species specific, it is likely that closely related species will perform the behaviour in a similar fashion with only minor variations. It is these similarities that are important in phylogenetic systematics.

The interesting facet of this termite behaviour is that the nest builders are all sterile. Therefore no nest building habit or structural modification of a sterile caste acquired during ontogeny could be transmitted to the succeeding generation. Emerson (1938) found that there was a strong correlation between behaviour of building the nests and the known morphological relationships between species. He also stated that the nests often furnish much better material for comparative studies than do the termites themselves (Emerson 1938). Interestingly, both of these 'phylogenies' (morphological and behavioural) were constructed using the traditional system of classification (section 1.3). No characters are given, no data matrix is given and the analysis of the relationships between species seems to be based on intuition.

There have also been behavioural studies on caddisfly case building behaviour. Ross (1964) studied 22 families of caddisflies and found that the evolution of home-making behaviour in Trichoptera
can be "explained harmoniously with the morphological indications of phylogeny" (Ross 1964). Again, this analysis appears to be based on intuition, since no characters are described or states given.

Spider web construction has been well studied by Eberhard (1982) and Coddington (1986). These two researchers have studied the actual behaviour and used this information in a phylogenetic analysis. Eberhard (1982) analyzed 6 families of orb-weaving spiders. Ten behavioural characters were used in this phylogeny. The results indicate that although some aspects of web design might be evolutionarily non-conservative, that the method of building the webs are conservative and therefore useful in determining relationships.

Coddington (1990) analyzed the architecture of web building spiders (Araneae: Araneomorphae; Orbiculariae) from 19 genera and found that his data support the existing, strictly morphological, phylogenies. Coddington used 11 behavioural characters as well as 50 morphological characters to produce his phylogeny. In an earlier work (1986) he gives a general description of web construction, and then gives comments on specific behaviours which he uses in his phylogeny. Coddington (1986) uses seven behavioural characters along with 17 morphological characters to reconstruct the phylogeny for 'non-orb weaving' ogre-faced spiders. In both these papers, Coddington (1986, 1990) used a cladistic approach to reconstruct the phylogeny.

Eberhard (1982), Coddington (1986), McLennan (1988) and Prum (1990) are the only authors that thoroughly describe the behaviour of their study organisms, give data matrices and phylogenies. In behavioural phylogenetics especially, it is extremely
important to describe all the intricacies of the entire behaviour precisely for proper definition of characters for phylogenetic analysis (Arntzen and Sparreboom 1989).

2.2.4 Behavioural Phylogenetics for Black Flies

The black fly cocoon spinning behaviour is innate, as the black fly larva has only one chance to build its cocoon. Individuals within a species spin cocoons so similar that the cocoon is used as a morphological character for identification of the pupa. It is proposed that the stages of cocoon spinning behaviour will prove to be excellent characters for constructing a phylogeny for this group.

In this study of black fly cocoon spinning behaviour the actual stages in the process of spinning the cocoon will be the characters used for the phylogenetic analysis, as opposed to studying the end product. In the research done on architecture, the behavioural characters have always been used in conjunction with morphology. This project will reconstruct the phylogeny for black flies using behaviour alone, and then compare the result with the existing morphological and chromosomal phylogenies.

2.3 PREVIOUS DESCRIPTIONS OF THE BLACK FLY COCOON SPINNING BEHAVIOUR

In order to use the stages of cocoon spinning behaviour as a group of characters for phylogenetic reconstruction, the behaviour must first be described thoroughly. This will result in a number of characters, which actually describe the behaviour itself. To date a thorough description of black fly cocoon spinning behaviour has not been published.
The topic of cocoon spinning in black flies has been addressed by only five researchers, beginning in 1923 and extending to 1966 (with a review in 1990 by Crosskey).

One interesting aspect of black fly cocoon spinning behaviour is that it is actually the pharate pupa, which outwardly resembles a larva, that spins the cocoon (Hinton 1958). (See section 1.1).

2.3.1 Pre Cocoon Spinning Behaviour

The site of cocoon building varies among species. Some will build the cocoon wherever they find themselves, whereas others will move around to find a suitable site (Crosskey 1990). When larval migration occurs, most tend to migrate to areas where the flow is less laminar, including the undersides of stones, cracks or depressions in rocks or the downstream sides of boulders (Peterson 1956; Crosskey 1990). Crosskey (1990) has suggested that this movement to more turbulent water is necessary for proper pupal respiration.

Once the larva has found a suitable location it has been noted that the larva begins the behaviour by “bending back and applying its mouth parts to a certain point on the support near its posterior end, then suddenly pulls its head away with some force” (Wu 1931). The purpose of this behaviour is supposedly to check the suitability of the substratum (Burton 1965), or to both check and clean the substratum prior to initiating the spinning process (Wu 1931; Peterson 1956; Crosskey 1990). Wu (1931) is the only author to have observed the duration of this process, stating that it may last longer than one minute.

The larva is attached to the substrate by an posterior circlet of hooks embedded into a pad of silk placed on the substrate. The
anterior end of the larva is directed downstream, although at times it may be at a slight angle to the current (Crosskey 1990). Regardless, the larva must build the posterior end of its cocoon behind itself, for the most part and therefore, must work against the current.

Previous researchers (Tonnoir 1923; Puri 1925; Wu 1931; Peterson 1956; Burton 1965) describe the pre-spinning behaviour in a similar fashion, after this, however, the descriptions of the actual cocoon spinning behaviour are quite different. Different species of the genus *Simulium* were being studied in all cases, though, which could lead to differing analyses. Tonnoir (1923) studied *S. tillyardi*, Puri (1925) observed *S. nölleri*, *S. erythrocephalum* and *S. aureum*, Wu (1931) studied *S. venustum*, Peterson (1956) investigated *S. vittatum*, and Burton (1965) analyzed *S. damnosum*.

Peterson (1956) made his observations using a binocular microscope with the larvae in the streams. While Tonnoir (1923) and Burton (1965) brought the larvae back to the lab and analyzed the behaviour there with the larvae in a beaker of moving water. It seems obvious from reviewing these papers, that these methods were not adequate to yield detailed results.

### 2.3.2 Cocoon Spinning Behaviour

Following is a brief summary of the cocoon spinning behaviour as described by each of these authors in chronological order.

Tonnoir in 1923 studied the cocoon spinning behaviour of *S. tillyardi*. His description is the most detailed of any that have been published to date. He actually determined that there were different stages in the cocoon spinning behaviour. *S. tillyardi makes* a circular
cocoon typically on the undersides of leaves, with its opening in a downstream direction.

Tonnoir (1923) noted that the larva never detaches during the spinning process. According to Tonnoir, after it has found a suitable location, the larva lays down six masses of silk around its abdomen, three on each side. This is repeated several times on each side. It is during this stage that the collar begins to be formed. In order to cross from one side to the other it moves in a half arc in front of itself. Later in the stage the larva pulls strands of silk from one mass of silk laid down earlier, to the collar at the front, and repeats this motion for each mass several times. There are a few strands of silk laid down from the collar to the most downstream extension of the larva.

In the next stage, the larva goes inside the cocoon by bringing its head underneath the collar with the mouth facing dorsally. This appears to be when the larva spins the majority of its cocoon. It finishes the collar and firmly attaches the cocoon to the substrate on both sides, crossing over many times using this same method.

Tonnoir (1923) states that the larva continues to spin the inside of the cocoon in a random or irregular fashion, but admitted that this was difficult to see. According to Tonnoir, the larva stops passing its head under its body but takes the reverse [over its back] position. During this spinning stage, it is concentrating the spinning on the floor of the cocoon, in order to support and hold the new pupa to the bottom during its maturation.

In 1925 I.M. Puri observed and wrote a brief description of the behaviour for three species, *S. nölleri*, *S. erythrocephalum* and *S. aureum*. He observed similar behaviours for all three species. It
appeared to Puri that the fibres forming the cocoon were laid down at random in an irregular network.

He outlined the behaviour as follows: "a) the larva at first spins a loose network of threads on the substratum between various points laid down at random on the flat surface just behind it, b) continuing, it spins threads passing dorsally over its body, whereby the cocoon becomes clearly defined as a loose basket, c) it now frees itself and becomes fixed at the cocoon-opening and thickens the network from the inner side".

Wu (1931) studied *S. venustum* and found that his observations did not coincide with Puri (1925) completely, in that the larva never changed its attachment during the spinning process. Wu (1931) described the behaviour as beginning with the larva bending back to place a loose network of silk just posterior to its point of attachment (p.o.a). Then it works "forward along the side of its body until the bend is about at its anterior third" (Wu 1931).

According to Wu (1931) the larva then fixes the silk to this support and twists so that its ventral side is outward and upward, while producing a strand of silk across the top in an arc-like fashion to a similar point on the other side. It repeats this procedure to form the rim of the future cocoon. The two ends of this arc are the extremes that the larva can reach. The limit that the larva can reach upstream becomes the apex of the cocoon. The larva then spins more threads along the sides and dorsally over its body, and the cocoon takes its shape. Lastly, work is completed at the closed end of the cocoon (Wu 1931).
Peterson (1956) made a detailed description of the behaviour of *S. vittatum*. Before it begins the actual spinning of the cocoon it lays down masses of silk in particular locations. According to Peterson, the first mass is placed as far downstream as possible. Then, with a strand of silk, moves to the left side of the p.o.a and lays down a smaller mass of silk. The thread is then moved as far as possible posteriorly (upstream) and a mass laid down (apex mass) in line with the first mass. The thread is then continued to the right side of the larval attachment, and a mass laid down. Finally, a strand connects this final mass to the first, downstream mass.

After this framework is made a net-like structure is spun, just downstream to where the rim of the cocoon will be located. This is done by connecting strands from the longitudinal threads on one side to those on the other side in front of the larval p.o.a (Peterson 1956).

According to Peterson (1956) the larva then starts to spin the cocoon. It starts by stringing threads from one lateral mass upward and dorsally over its body to the corresponding mass on the opposite side making the rim (as described also by Wu (1931)). Next there is a series of threads placed from the arch to the upstream mass of silk where they are attached closely together to form the upstream apex of the cocoon. From the apex the larva works forward spinning threads and attaching them from side to side connecting the right and left upstream longitudinal threads. Peterson (1956) describes this stage as follows: the larva “twisted and turned spinning silk from side to side and forward and backward soon giving shape to the cocoon”. The larva then worked under the cocoon along both sides in order to add
an inward-projecting thin, flat mass of silk placed directly on the substrate along the length of the cocoon.

For the completion of the cocoon the larva begins to spin the floor. This starts at the upstream end and proceeds in the downstream direction. Threads are attached from the apex of the cocoon to the inward-projecting flattened mass from each of the two sides. This loose network extends about one quarter of the length of the cocoon. With continued spinning the floor ends up covering about half the length of the cocoon (Peterson 1956). This is contrary to the description given by Wu (1931) in which the floor was laid down first.

Burton (1965) studied *S. damnosum* almost ten years later than Peterson, but the cocoon spinning behaviour is not described in as much detail. Burton stated that the larva first lays down a pattern of threads in a random fashion. “The preliminary threads are applied anteriorly, laterally and posteriorly in an irregular pattern and form the sides, roof and posterior floor of the cocoon.”

Burton (1965) states that the floor is laid down initially with threads affixed longitudinally. These cross at acute angles, followed by threads which transverse and connect to form a coarse uneven network. The body “doubles up and rotates as it weaves, often twisting on its axis through 180 degrees or more”. Burton stated that the larva may detach itself after the basic network has been laid down.

After about 15 minutes the larva concentrates on the posterior end, weaving it minutely and closely. It appeared to him that there is a strong adhesive associated with the threads because of the strong
attachment of the cocoon to the substrate. This substance presumably fills the minute spaces between the threads, giving rigidity to the cocoon (Burton 1965).

The last description of cocoon spinning was provided by Crosskey (1990). His description is primarily a review of these other researchers' work, especially that of Peterson (1956). Crosskey (1990) describes the stages as 1) the encirclement of the body in a framework placing masses of silk and connecting them, 2) connection of the frame sides to make a cover over the abdomen. Then the larva moves inside this structure and 3) reinforces the cocoon ceiling and lastly 4) the floor and adhesive skirting is spun.

There are a number of points upon which the authors cannot agree. One is whether the larva detaches itself during the spinning process. Most say that the larva does not detach (Tonnoir 1923; Wu 1931; Peterson 1956; Crosskey 1990), while the others state that it does (Puri 1925; Burton 1965). They also disagree on the sequence of spinning, in particular, whether the floor is built first (Wu 1931; Burton 1965) or last (Tonnoir 1923; Peterson 1956; Crosskey 1990) in the process.

The variation in these descriptions and the lack of detailed explanation of the movements of the larva make it difficult to understand exactly how the cocoon is spun. The whole process has been described to take a varying amount of time ranging between 38 and 70 minutes. However, as mentioned previously all these authors were studying different species and the variation in spinning times could be the result of interspecific variation or varying conditions under which they were studied.
2.3.3 Post Cocoon Spinning Behaviour

After the cocoon has been spun, the larva must remove the larval skin and get inside the cocoon. This behaviour is visible, even with the naked eye and most researchers described the behaviour in a similar fashion.

The larva doubles back on itself to form a tight U shape, back to back, with head and posterior sections outside the cocoon (Tonnoir 1923; Wu 1931; Burton 1965; Crosskey 1990). It then rotates for 10-15 minutes. A slit then appears along the lateral and the posterior border of the fronto-clypeus and lengthwise to the mesothoracic region. After this the pupal head and thorax begin to emerge (Tonnoir 1923; Wu 1931; Burton 1965; Crosskey 1990). The pupal gills start to uncoil as soon as they are immersed in water and quickly expand to their characteristic pupal form (Crosskey 1990). The pupa will then wriggle back into the cocoon for a few minutes before becoming stationary (Wu 1931; Crosskey 1990).

The duration of this behaviour was described by a few of the researchers. Wu (1931) stated that it took 25 minutes for the doubling up of the larva to the ecdysis and 50-60 minutes for the spinning, totaling 75-85 minutes for the whole process. Puri (1925) and Crosskey (1990) both state that the whole process from the initiation of spinning to the removal of the larval skin takes between 75-90 minutes.

It seems clear from this information that the whole cocoon spinning process in black flies has not been adequately described by any of these researchers. This is likely due to the lack of technology for allowing close observation of the process. Certain aspects of the
behaviour are visible under the conditions used by these researchers, while others are too complex and require the ability to watch the stages many times.

Many of these descriptions were merely casual observations of a small number of larvae. The use of this behaviour for phylogenetic reconstruction necessitates the thorough description of this behaviour, indicating not merely that the larva was moving "side to side" but rather, the exact steps and movements, as well as the duration of these steps for different species.

3.0 STATEMENT OF OBJECTIVES

There are four main goals which I wanted to achieve for my MSc thesis. First, it is necessary to understand thoroughly the behaviour of each species being studied. Therefore complete analyses of the behaviour for each species will be undertaken. Second, the behaviour must be broken down into its component parts (as much as possible) and characters designated for each separate component of the behaviour. Third, a number of species must be analyzed in this fashion and a cladistic analysis of the designated characters undertaken to produce a phylogenetic tree. The fourth goal is to then take these constructed phylogenies and compare them to existing phylogenies and attempt to explain any incongruencies.

4.0 MATERIALS AND METHODS

The present study was undertaken from May-July, 1993 and 1994, at the Wildlife Research Station in Algonquin Provincial Park,
Ontario, Canada. (45°34'N,78°41'W). Additional data were collected in Alberta, Nova Scotia and South Carolina (see Table 2).

4.1 COLLECTION OF LARVAE

When a breeding site was located that had larvae present, it was visited and the larvae removed from the substrates using forceps. In the present study all large larvae were collected. They were transferred to specimen cups containing a small volume of stream water. These were placed in a cooler on ice for transport back to the lab. While at the stream, the water temperature was measured and the flow tested by determining the time required for a light floatable object to travel one metre in the stream.

In the lab the pharate pupae were separated from the immature larvae under the microscope on the basis of the presence of dark respiratory histoblasts on the pharate pupae. Pharate pupae were identified to species using larval headspot patterns and other morphological characters found in keys for *Simulium* by Currie (1986) and Wood et al. (1963). The pharate pupa superficially resembles a larva, so the term larva will be used for the remainder of this section.

4.2 NOMENCLATURE USED

In this thesis *Simulium* and *Eusimulium* will be given status as separate genera. Presently they are both in the genus *Simulium*, with separate sub-generic status. This will be changed in the upcoming book *The Black Flies of North America* (Dr. D.C. Currie, University of Toronto, pers. comm. 1995). The separation of these two genera has been used by authors from Europe for decades as is apparent in Rothfels (1979). It was decided that this thesis would take this new approach and recognize two separate genera.
4.3 THE FLUME

The flume (artificial stream) (Figure 2) in which the larvae were placed is made of Plexiglas and has two chambers, one at either end, and a raised (stream) surface connecting the two chambers. A pump and pipe connecting these two chambers, force the water from the downstream chamber back to the upstream chamber, and then the water flows over the stream surface to complete the cycle. The pump was adjustable to control the rate of flow over the surface. A fan was required on top of the pump to reduce the effect of the heat that was produced by the pump on the water in the flume.

The temperature and flow of the stream where the larvae were collected was maintained as closely as possible. There were two flumes used in this experiment. This was necessary to film different species at the same time as well as to maintain two different conditions depending on the requirements of the species being filmed. One of the flumes was equipped with a cooling unit that consisted of a peltier coil, power supply (to run the peltier coil), fans and Styrofoam insulation. This was required for species that could not tolerate warm water temperatures. The peltier coil was attached to the pipe transferring water to the upstream chamber of the stream, the fans removed the heat produced by the peltier coil.

When placing the desired larvae into the flume, the water level was higher than the stream surface and the water stationary (i.e., the pump turned off). This allowed the larvae to attach themselves to the substrate before the water flow was turned on. Once most individuals had attached, the water was turned on and the flow increased gradually until the desired rate was reached.
Figure 2: The flume and recording set-up for study of the cocoon spinning behaviour in black flies. Set-up includes: the flume, video camera, macro lens, tripod, television and vcr.
4.4 RECORDING TECHNIQUES

A JVC® video camera was used to record the larvae spinning their cocoons. This camera had an attachment Sigma® macro lens which enlarged the larva being filmed to fill a television screen. The video camera and macro lens were on a tripod and were directed downward onto the stream surface of the flume. It was then attached to a standard VHS video recorder and television for observation during the taping process. Standard VHS tapes were used on extended play to allow for 6 hours of taping per tape. The video playback system allowed for counting crosses, timing stages and close observation of the spinning behaviour during each stage.

When a larva was preparing to spin its cocoon, it would tend to move around in the flume. The tripod was not attached to the floor and therefore the video recording system was portable and the larva could be followed. When the larva started to clean one spot for a few minutes it was found that it would not likely move again. This was when the recording started. The recording stopped after the pupa wriggled into its cocoon and was settled for a few minutes.

4.5 MARKING AND REARING TECHNIQUES

For each pupa that was recorded a designated number was assigned and marked on the underside of the stream surface; this corresponded to the number on the video cassette. The pupa was left in the flume for about 48 hours to mature and to ensure that the silk had hardened completely. The pupa in its cocoon was then removed from the flume, checked to ensure that the cocoon corresponded to cocoons found in the literature for the particular species, and placed on a moistened piece of filter paper in a 1.5 mL Eppendorf®
microcentrifuge tubule. This process was done in order to rear the black fly to an adult, for identification to species (Hunter et al. 1994). Some species of black fly are readily identified using the larval and pupal stages using morphological keys (Currie 1986; Wood et al. 1963). However, some require reared adults for positive identification, e.g., members of the S. venustum/verecundum complex. These can be identified using adult morphological keys (Hunter 1990). The numbered pupae and/or adults were all identified to species.

4.6 ANALYSIS

Each individual was analyzed by watching the playback on a standard VHS video recorder and television set. First the larvae were watched in chronological order, and later they were watched again by groups of individuals all identified as the same species. Each was watched from the time that it started spinning until the pupa broke through the old larval skin and started to wriggle down into the cocoon.

In the process of the analysis, it was noted that there were six distinct stages of spinning in most species. Each stage was timed in seconds, and the number of times the larva switched from one side to the other was counted. All behavioural units during the spinning process were noted. All phylogenetic analysis was performed sensu Hennig (1966).

4.6.1 Codes for the qualitative phylogeny

This phylogeny is based on 21 characters for 20 species. The characters were chosen by observing the qualitative behaviours of cocoon spinning for all species. All the characters will be discussed in detail in section 5.3.1.
4.6.2 Codes for the quantitative phylogeny

There were two methods undertaken to reconstruct phylogenies using only quantitative information. The first method involved the gap coding technique (Goldman 1988). This quantitative tree comprised 10 species each represented by 6 individuals. The ratios of duration of each stage/total spinning time (6 characters), the ratios of crosses for each stage/total number of crosses (6 characters) and the ratio of time spent breaking the larval skin/total time from start of spinning to ecdysis, were the characters used in this phylogeny.

These ratios were then arcsin transformed, which must be done with all ratio or percentage data (Zar 1984), in order to make the data normally distributed. The means and standard deviations of each of the 13 characters for each species were calculated. A pooled in-group standard deviation was calculated by averaging the standard deviations for all species and each character. The codes for the phylogenetic analysis were formulated using the gap coding technique for continuous data (Goldman 1988). Basically, the means for one character are placed in order from smallest to largest. The pooled in-group standard deviation is then added to the smallest mean. This gives a total, if any means for other species fall within the total, the corresponding species is given a code of 0. Of the means that fall into this initial total, the standard deviation is added to the largest mean in the group. Again, any mean falling within this new total, the corresponding species is given a code of 1. This is repeated until all the means, and therefore, species, are given a code. If no means fall within a given total, then the code number is skipped and the
standard deviation is added to that total until a mean falls into the total. This was repeated for all 12 remaining characters.

The second method employed involved computing the means and confidence intervals for the ratio of duration and crosses for each of the six stages. This yielded 12 data points (the ratios of duration of each stage/total spinning time (6), the ratios of crosses for each stage/total number of crosses (6)). These were then arcsin transformed, as explained earlier.

The method of coding was as follows: mean of stage 1 significantly less than mean of stage 2 = 0, mean stage 1 less but not significantly less than mean of stage 2 = 1, mean of stage 1 equal to mean of stage 2 = 2, mean of stage 1 greater, but not significantly greater than mean of stage 2 = 3, mean of stage 1 significantly greater than mean of stage 2 = 4. Significantly greater and less than were determined by confidence intervals not crossing (See Appendix 1, Figure 1, stages 3 vs. 4). Insignificantly greater or less than were determined by having one confidence interval of each stage crossing, but neither of these crossed the mean of the other stage (See Appendix 1, Figure 1, stages 4 vs. 5). Equality was determined by confidence intervals that cross the means of the stages being compared (See Appendix 2, Figure 1, stages 2 vs. 5).

These codes were then applied to all combinations of the stages 1-6 and 7-12. For example: 1 vs. 2, 1 vs. 3, 1 vs. 4, 1 vs. 5, 1 vs. 6, 2 vs. 3, 2 vs. 4 etc. There were 28 characters arising from this analysis (autapotypic characters were excluded).

4.6.3 Analysis of the phylogenies
The phylogenetic trees discussed herein, have all been produced in the same manner. MacClade was initially used to set up the data matrix. All species and characters were placed in this program. MacClade (Maddison and Maddison 1992) assumes that there are only 2 character states for the weighting procedure. Therefore, if one character has 2 states and another has 4 states the latter will be given twice as much weight as the former. In order to compensate for this, the character with 2 states would get a weight of 2 and a character with 4 states would get a weight of 1. In reality, therefore, all characters were weighted equally. All the characters were assumed to be unordered, because the order of the evolution of the behaviours is not clear. In the gap-coding technique (Figure 12), however, the characters must be ordered (Maddison and Maddison 1992).

The polarity was assumed to coincide with Figure 15, thus, Prosimulium was considered the outgroup and the remainder of the species were considered to be monophyletic. However, if a character was not present in Prosimulium, the polarity could not be inferred, therefore the character was run as an unpolarized character. The polarity was determined *a posteriori* based on the other characters in the matrix. Species were coded with a '?' if the character was not present, or there was insufficient information to reliably give it a code.

Characters that had inapplicable taxa were also run with each taxa considered as autapotypic, this gave more equally parsimonious trees, but they had the same consensus trees. Since the trees produced using missing data were much easier to decipher, and did not change the topology of the consensus tree, these trees were used in the analysis. Characters 12+13 and 15+16+17 were also run as
multistate characters and again there were more (51) equally parsimonious trees, but they had the same consensus trees, therefore the matrix found in Appendix 1 Table 1 was used.

The matrix was then run through PAUP 3.1 (Swofford 1990). In this program an exhaustive search was performed. When the most parsimonious tree(s) was (were) found, each tree was observed and compared to the phylogeny in Figure 15. A 50% majority rule consensus tree was used to determine which areas were problematic in the tree. Areas that were unresolved were left as such because it was not possible for the data to resolve these conflicts. Using MacClade 3.0 (Maddison and Maddison 1992), the tree that most resembled the morphological and cytological tree (Figure 15) (aside from the unresolved areas) was reconstructed and each character was viewed using the trace tool to determine where each character had arisen on the tree. The numerical analyses used for phylogenetic analysis are the consistency index and the retention index.

_Ectemnia invenusta_ was added _a posteriori_ in the MacClade program and placed at the most basal position on the tree where the addition of this species did not reduce the consistency or retention indices.

**5.0 RESULTS**

**5.1 DESCRIPTION OF THE COCOON SPINNING BEHAVIOUR OF _Simulium vittatum_**

Although _Simulium vittatum_ is actually a complex consisting of two sibling species (with III-1 most common in our study site, Algonquin Park), it was chosen for the base line description of cocoon spinning for several of reasons. It is one of the species whose cocoon
Figure 3: Photograph of *S. vittatum* cocoon. A) Abbreviations: ap = apex; c = collar; ant = anterior; pos = posterior; poa = point of attachment; t = top; w = wall. B) the cleaning stage of *S. vittatum* with pads of silk present on the substrate, C) the complete cocoon of *S. vittatum* indicating the position of the pads in relation to the finished cocoon.
spinning behaviour was described by Peterson (1956), thus allowing for some direct comparisons. It is widespread and can tolerate diverse conditions (Peterson 1981), thus allowing many individuals to be recorded successfully and reared for identification. Most importantly, it was essential to choose a species that had the basic stages of cocoon-spinning that were easy to see and describe, so that this description would be a suitable model for future analyses. All species that have been recorded in either the genus *Eusimulium* or *Simulium* have the spinning stages described in this paper, although some make more complicated cocoons. It was determined that a simple slipper shaped cocoon would be the best for a base line description, and that species with more complex cocoons will have those behaviours described when necessary.

5.1.1 Pre-Spinning Behaviour

This study is an analysis of 17 individuals, all but three of which were complete recordings. The terminology that is used in this description, and the area of the cocoon that it represents, is found in Figure 3.

The larva first places down a pad of silk and attaches its posterior circlet of hooks to the point of larval attachment (p.o.a.) (Figure 4). The larva remains attached in this location until the spinning process is completed. When the cocoon is completed, this p.o.a. is approximately half way between the anterior and posterior ends of the cocoon. The posterior end of the cocoon is upstream of the p.o.a., and therefore the larva must spin behind itself, against the current. This area must be free of debris before the larva can begin
spinning. This requires an extended cleaning period, which can last for an hour in some individuals.

There are two forms of cleaning that have been observed: scraping, where the mouthparts are in motion but never leave the substrate; and tugging, where the larva engages the substrate with its hypostoma and quickly raises its head.

A larva cleans the surrounding substrate by bending itself in a 'U' position and moving progressively upstream, combining both scraping and tugging. If a large pad of silk is present (from previous individuals moving through the area), the latter method of cleaning is used. The larva switches many (approximately 20-60 depending on the amount of time spent cleaning) times from side to side, crossing at the front (similar to the 'front' cross stage of spinning (section 5.1.2.1) during the cleaning process. The longest videotaped cleaning observed was 45 minutes.

Before spinning, a *S. vittatum* larva lays down silk pads downstream from its p.o.a. of the posterior circlet of hooks. The larva must bend its body to do this because the pad is not placed at the extension of the larva's body, but slightly closer to the p.o.a. This pad of silk will become the anterior-most portion of the cocoon. Most *S. vittatum* (11 of 14) have been observed laying a second pad to the side of the initial pad (Figure 4).

After the larva has cleaned both sides many times it will initiate the spinning of the cocoon. It works its way up one side cleaning, as described above, and when reaching the apex of the future cocoon it will draw a single strand to the front and terminate the strand on one
of the pads of silk laid down previously. The larva then typically adds to the initial pad before moving up the opposite side.

5.1.2 Spinning behaviour

5.1.2.1 Stage 1: Initial Structure and Front Cross

The larva bends itself into a U, reaches upstream about 1/4 of its length past the p.o.a., and touches the substrate, attaching a strand of silk. It then moves in a downstream direction pulling the strand over its back and terminates the strand at the front on the same side (Figure 4b).

It then changes side, using the front cross. That is, it straightens itself out at the front and continues in a half arc motion to the other side. It then bends itself into a U on this side and repeats the pattern described above. From here on, the larva lays down more than one strand per side before crossing to the other side. After attaching the first strand at the front the larva will then reach farther upstream and place another strand, pulling it around its back to the front, as before. Therefore, the first strand is initiated just near the p.o.a., the second is initiated farther upstream, the third even farther upstream, etc. Each progressive strand is pulled farther across the back. Typically 3-5 strands are laid down per side before performing a front cross to the other side. The larva crosses 12.6 +/- 2.4 times for a total of 270 +/- 43 seconds (n=14).

Near the end of this stage the larva will concentrate more on the sides adjacent to itself. The latter strands of each cross during this portion of the stage are placed on the opposite side of the p.o.a. Therefore, if spinning from the right side, it will place the silk on the left side of the p.o.a. and bring the strand over its back to the right
side at the front. This is a gradual progression. Early in the stage the larva extends as far upstream as possible, but at the end it concentrates the spinning at the anterior end of the future cocoon. The spinning at the end of the stage appears to make a tent-like structure ('pupation tent' sensu Crosskey (1990)).

At the completion of this stage the larva appears to have two lines of silk parallel to itself, one on each side, and the pupation tent anteriorly.

5.1.2.2 Stage 2: Walls and 'S' Cross

This stage begins when the larva twists itself, at the front, into the shape of an 'S' and tucks its head inside the initial structure it has built (Figure 5). For the first few S crosses the larva lays strands of silk from its mid section to the front 4-5 times before tucking its head under to go to the other side. Henceforth the cocoon is spun from the inside. The concentration during this stage is the initial building of the walls of the cocoon.

The larva spins each side in a series of step-like moves. It starts by moving its mouthparts almost to the p.o.a. and then pulls a strand in the downstream direction approximately 1/4 of its body length. The next step it starts beyond the p.o.a. and again pulls the strand in the downstream direction. The larva repeats this procedure 5-6 times on each side, each time progressing closer to the future apex (or most upstream end) of the cocoon structure. The larva twists while laying down the strands to cover the top of the cocoon as well as the walls. During this stage the threads are concentrated near the front of the cocoon. This stage is easily distinguished because of the "S" cross at
the front which occurs 9.6 +/- 2.3 times. The stage lasts for 352 +/- 105 seconds (n=16).

Near the end of this stage the larva places strands down as before, but when pulling anteriorly, it pulls the strand distinctively over the back. Therefore the strand is placed in a diagonal direction from the wall farther upstream, to the middle of the top of the future cocoon, downstream. The larva returns to the wall at the front and performs another "S" cross. At the completion of this stage the pupation tent and the anterior half of the cocoon are visible.

5.1.2.3 Stage 3: Walls and Back Cross

This stage begins when the larva places a strand and pulls it diagonally over its back (Figure 6b, part a), but instead of falling to the same side at the front, continues diagonally to the other side, and starts spinning up the other side (Figure 6b, part b). This is the back cross, in which the larva crosses over the back from the apex of the cocoon to the front on the opposite side. The head of the larva does not extend outside the mouth of the cocoon after this point.

Spinning is concentrated on the walls during this stage as well. The larva works in a fashion similar to the previous stage, moving upstream and pulling a strand in the downstream direction but during this stage the strands are laid down in the shape of a sideways V. There are three different methods of spinning during this stage. When the stage begins the larva initiates the strand of silk on the wall of the cocoon and rolls onto the floor and then back to the wall, in a sideways V. This type of spinning is repeated 4-6 times until the larva reaches the apex of the cocoon, where it will back cross to the other side. The second type of spinning is the wall-floor connection.
Figures 4-9: A) Photographs of *S. vittatum* representing the first step (a) of the corresponding schematic diagrams B) Computer-generated schematic diagrams of *S. vittatum* spinning its cocoon. The diagrams were composed on an illustrator program using scanned photographs. Headspot patterns indicate the dorsal surface of the larva.

Figure 4: The building of the 'initial structure' of the cocoon a) larva extended past the p.o.a. and initiating the strand of silk, b) larva pulling the strand of silk over its back, c) the termination of the strand of silk at the lateral side of the larva.

Figure 5: The 'S' cross a) the larva bent in a 'U' shape with the head pulled anteriorly, b) the larva in the shape of an 'S' with head crossing over towards the other side, c) the larva tucking its head between itself and the substrate to arrive on the other side.

Figure 6: The 'back cross' stage a) a lateral view of the larva after building the wall on the left side of the cocoon, b) the larva at the anterior end of the future cocoon.
This is done by placing the mouthparts at the point where the wall of the cocoon meets the substrate where the larva is spinning. The larva will move upstream and pull strands downstream, but this is done quite slowly in a straight line (i.e., there is no V type movement). This motion is repeated 6-10 times to reach the apex of the cocoon. In the last type of spinning for this stage, the larva places the strand on the wall, twists slightly over its back and twists back to the wall on the same side. It repeats this motion 7-15 times, moving progressively farther upstream each time before pulling all the way back to the front on the opposite side. The larva tends to make fewer, longer pulls at the beginning of this stage and more, shorter pulls by the end. The larva crosses 12.0 +/− 1.8 (all types of spinning included) times for a duration of 571 +/− 166 seconds (n=17).

By the end of this stage the form of the cocoon is clearly visible. The walls are strong and the top of the cocoon has been laid down. Near the end of this stage the larva starts to pull the strands from the apex to the middle of the front of the cocoon, as opposed to pulling the strand directly to the opposite side. It then spins on the collar of the cocoon as it falls to the opposite side. For timing purposes, this stage starts at the first back cross, where the larva goes to the front on the opposite side and ends when the top-pull front cross stage starts.

5.1.2.4 Stage 4: Top and Pull Front Cross

The transformation to this stage is the most difficult to observe but there are two criteria which must be met. First, the larva, after completing the spinning of one side, must pull a strand from the apex of the cocoon all the way to the front, middle of the cocoon (Figure 7). Each time the larva pulls to the front it remains at the front for about
10 seconds to spin the collar. Second, the larva must spin on the top of the cocoon (Figure 8).

It begins this stage working up one side in a fashion similar to the ‘walls-back cross’ stage. Once the larva passes its p.o.a. it then starts a strand from the center of the top and pulls back towards the front in a sideways V motion again. The larva alternates lateral directions of the strands. One strand crosses the median of the top of the cocoon, thereby spinning onto the other side and then comes back to the original side. It then moves its mouthparts slightly downstream before laying down the next strand on the original side.

It starts at the center of the top of the cocoon. Then the larva moves to the wall and back to the center. This combination is repeated moving closer to the apex each time always pulling in the downstream direction.

Both these criteria must be met for the stage to be said to have started. The stage begins, for timing purposes at the cross previous to the first time the larva spins on the top of the cocoon.

There are 5.9 +/- 1.0 (n=17) crosses during this stage and it lasts for 317 +/- 80 seconds (n=17). Near the end of this stage the larva spends less time spinning the collar, and gives preference to either the left or right half of the top of the cocoon. By the end of this stage the cocoon appears, in dorsal view, to be complete.

5.1.2.5 Stage 5: Bottom and Pull Cross

The initiation of this stage also has two criteria. First, the larva pulls from the apex, as before, but now stops just short of the collar of the cocoon, and does not spin on the collar at all. Second, the larva
Figure 7: The 'pull front' cross a) the larva at the apex of the cocoon on the left side, b) the larva at the anterior end of the cocoon. The larva spins the collar at this stage, starting from the middle and working over to the opposite side.

Figure 8: The spinning of the top of the cocoon during the 'pull front' stage a) the ventral surface of the larva near the apex of the cocoon, b) the larva pulling a strand of silk downstream to the left side of the cocoon, c) the larva continuing the strand of silk progressively downstream, but moving back toward the middle of the cocoon. The end product of this movement is a strand of silk placed in a sideways 'V'.

Figure 9: The reinforcement stage a) the larva at the apex of the cocoon, b) the larva pulling a strand onto the bottom of the cocoon, so its dorsal surface is again visible.
must spin onto the bottom of the cocoon. The abbreviated pull is usually the indicator that this stage has begun.

When the larva is working its way toward the apex during this stage, it still spins in a sideways V motion and will again alternate lateral directions, but this time the strand is initiated on the wall. This means that one time the V will cross over the midline on the top and the next strand will cross the midline on the bottom.

It is quite easy to determine that the larva is spinning the bottom because throughout most of the spinning process only the larva's lateral sides or the ventral surface have been visible. However, in this stage the dorsal surface is visible as well.

This pull cross is performed 5.9 +/- 1.7 times during the 317 +/- 109 seconds (n=17) of its duration. This stage starts at the first pull that does not quite reach the collar, as long as the larva then goes on to spin the bottom on that side. Near the end of this stage one strand will go onto the wall and the other will go onto the bottom. Therefore, the larva is concentrating less and less on the top of the cocoon. Also, the larva will concentrate on the posterior 1/3 of the cocoon.

5.1.2.6 Stage 6: Reinforcement and Flip Cross

This stage begins when the larva reaches the apex of the cocoon and pulls strands in the downstream direction. The difference is that the strand is pulled in only one lateral direction in the shape of a sideways V and it is always on the bottom of the cocoon. The larva will start at the apex for each strand laid down during this stage and twist underneath to cross the median to the other side and then cross back moving toward the front of the cocoon (Figure 9). Then it will move all the way to the apex again and repeat the motion. The larva
switches sides by twisting, usually spinning one strand on the top in the process, to the other side. It performs $4.9 \pm 2.0$ of these crosses in $216 \pm 74$ seconds ($n=17$) of spinning. This stage ends when the larva releases its posterior circlet of hooks.

The total time required to spin the cocoon is $33.7 \pm 7.0$ minutes ($n=17$).

5.1.3 Post Spinning Behaviour

5.1.3.1 Release

The larva releases its anal circlet of hooks and enters the U position. This U position is different from that previously described because the larva is facing in the other direction. This time the head and posterior portions are outside the cocoon and the midsection is inside the cocoon.

However, it takes $75 \pm 29$ seconds before it starts to twist on itself, in a regular fashion. It appears to spend this time getting properly aligned and possibly lays down some final strands of silk at the anterior end of the cocoon. After this stage, however, the mouthparts no longer move. The larva will either have its head facing the left or right wall of the cocoon.

5.1.3.2 Breaking the larval skin

This stage is initiated when the larva starts to twist on itself, with the dorsal surface of the thorax pressed to the dorsal surface of abdomen, in a rhythmic fashion. The larva turns on itself $67.6 \pm 7.5$ times for a total of $587 \pm 81$ seconds. This stage is terminated when the larval skin is broken and the pupa starts to wriggle out of the skin.
Table 2: A list of the species used in this study, collection sites and dates. This is an incomplete list of possible sites and dates where these species are available for collection, as only dates of actual collection are noted.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>NUMBER OBSERVED</th>
<th>COLLECTION LOCATION</th>
<th>COORDINATES</th>
<th>COLLECTION DATES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prosimulium</td>
<td>4</td>
<td>LaHave River, Nova Scotia</td>
<td>44°16' N 64°20' W</td>
<td>20/04/95</td>
</tr>
<tr>
<td>Cnephal dacotensis (Dyar and Shannon)</td>
<td>2</td>
<td>Costello Cr.</td>
<td>45°34' N 78°41' W</td>
<td>20/5/94</td>
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<tr>
<td>Stegoptera mutata (Malloch)</td>
<td>20</td>
<td>Chit Lake Trail</td>
<td>45°34' N 78°41' W</td>
<td>10/5/93 + 5/5/94</td>
</tr>
<tr>
<td>Ectenmia invenusta</td>
<td>3 partial</td>
<td>North Madawaska: Highway 60</td>
<td>45°34' N 78°41' W</td>
<td>3/3/94 + 2/3/95</td>
</tr>
<tr>
<td>E. gouldingi (Stone)</td>
<td>5</td>
<td>Lake Sasajewan Trickle</td>
<td>45°34' N 78°41' W</td>
<td>1/14/93 + 1/6/94</td>
</tr>
<tr>
<td>E. anatinum (Stone)</td>
<td>2</td>
<td>Highland Hiking Trail</td>
<td>45°34' N 78°41' W</td>
<td>6/6/94</td>
</tr>
<tr>
<td>E. congrearum Dyar and Shannon</td>
<td>15</td>
<td>Highland Hiking Trail</td>
<td>45°34' N 78°41' W</td>
<td>6/6/94</td>
</tr>
<tr>
<td>E. croxtoni (Nicholson and Michel)</td>
<td>10</td>
<td>Spruce Bog</td>
<td>45°34' N 78°41' W</td>
<td>1/6/94</td>
</tr>
<tr>
<td>E. craigi (Adler and Currie)</td>
<td>5</td>
<td>Highland Hiking Trail</td>
<td>45°34' N 78°41' W</td>
<td>6/6/94</td>
</tr>
<tr>
<td>E. caledonense (Adler and Currie)</td>
<td>12</td>
<td>Rock Lake Hill</td>
<td>45°34' N 78°41' W</td>
<td>11/5/94</td>
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<tr>
<td>E. rivuli (Twin)</td>
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<td>Kearney Lake Outlet</td>
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<td>29/4/94</td>
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<td>S. venustum Say</td>
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<td>45°34' N 78°41' W</td>
<td>19/5/94 + 24/5/94</td>
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<tr>
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<td>45°34' N 78°41' W</td>
<td>28/29/6/94</td>
</tr>
<tr>
<td>S. tuberosum (a) (Lundstrom)</td>
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<td>Lobstick River, Alberta</td>
<td>53°37' N 115°00' W</td>
<td>1/8/1994</td>
</tr>
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<td>24/6/94</td>
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<td>S. vittatum Zettersted</td>
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<td>2/5/1993</td>
</tr>
<tr>
<td>S. decorum Walker</td>
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<td>Davies Bog</td>
<td>45°34' N 78°41' W</td>
<td>25/6/93</td>
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<td>S. pictipes Hagen</td>
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<td>34°48' N 82°50' W</td>
<td>14/05/95</td>
</tr>
<tr>
<td>S. krebsorum Molton and Adler</td>
<td>6</td>
<td>Harmon's Mill Pond Outflow, South Carolina</td>
<td>33°58' N 80°49' W</td>
<td>16/05/95</td>
</tr>
<tr>
<td>S. dixiense (Stone and Snoddy)</td>
<td>6</td>
<td>Harmon's Mill Pond Outflow, South Carolina</td>
<td>33°58' N 80°49' W</td>
<td>16/05/95</td>
</tr>
</tbody>
</table>
Figure 10: Diagramatic representation of *Ectemnia invenusta* spinning the stalk where it resides and eventually spins its cocoon.
The entire process for *S. vittatum*, from initiation of spinning to removal of the larval skin, takes 44.8 +/- 7.9 minutes (n=14).

5.1.4 Other Species Studied

Nineteen species, besides *S. vittatum* were observed in detail for this study, as found in Table 2.

5.2 STOCK BUILDING AND COCOON SPINNING BEHAVIOUR IN *Ectemnia invenusta*

*Ectemnia invenusta* is an interesting species of black fly when studying cocoons, particularly because larvae build their own stalks on which they pupate. This species builds its own stalk in the larval stage where it feeds and rests. This stalk gets built by the larva bending forward in a ‘U’ shape (ventral surfaces toward each other) and placing a mass of silk down and attaching itself to this mass. It will then continue to lay down these masses until a small stalk has been built. The larva will then release itself and move almost to the end of the stalk. It will then add on to the stalk using a half-arc motion, building on one side of the stalk only. It will then release itself and, with mouth attached to the end of the stalk twist itself 180° to be placed on the opposite side of the stalk (Figure 10). It will again not move completely to the end, but keep a small part of the stalk in the groove of the ventral abdominal expansion. It will then add to the other side of the stalk again using the half-arc motion.

Intermittently, the larva will reinforce the entire stalk that it has built, by placing its mouthparts on the tip of the stalk and moving progressively down the stalk until it reaches the base. This sometimes
will involve bending the stalk, if it is longer than the body of the larva. The larva does not spin this stalk constantly, it will break and feed, sometimes for extended periods before returning to the spinning of the stalk.

5.3 DESCRIPTION OF CHARACTERS FOR PHYLOGENETIC RECONSTRUCTION

The following are descriptions of each of the 23 characters used in the final analysis (Figure 12), and the codes used for the analysis:

1) Cocoon foundation:

0) 2 surfaces. The larvae of some species will always spin their cocoons in a crack or corner. Individuals use both the wall and floor as attachments for spinning. The species that must have two surfaces to properly attach their cocoons to the substrate tend to spin sac-like cocoons.

1) 1 surface. The remainder of the species are able to spin in cracks and corners but also in open, flat areas. Individuals only use one surface for cocoon spinning.

2) Front strands:

This character refers to the type of strands used during the initial structure stage of spinning. All species studied performed the initial structure stage, therefore this was not used as a character (pleisotypic) For more information on the initial structure stage see section 5.1.2.1.

0) Transverse. The larva lays the strands of silk transversely in relation to the resting larva. At the completion of the front cross stage the initial cocoon is predominantly transverse to the
larva, and therefore also to the flow of water in the stream. These transverse strands are always spun posteriorly to the future cocoon, and there are usually transverse strands spun anteriorly to the future cocoon as well.

1) Longitudinal. The strands of silk are laid from a point posterior to the p.o.a., and pulled across the dorsal surface of the larva and terminated antero-laterally on the same side. This is repeated 3-6 times before crossing to the opposite side. This results in an initial structure that is parallel to the longitudinal axis of the larva, and to the flow of water.

3) Area of concentration during initial structure stage (Far/Near ratio):

During the ‘far’ portion of this stage the larva will reach posteriorly as far as possible and initiate a strand of silk there. It will then pull this strand anteriorly and terminate the strand on the same side. The posterior end of the initial structure is built during the ‘far’ portion of this stage. During the ‘near’ portion of the stage the larva will concentrate on the anterior portion of the future cocoon, rarely spinning strands much farther posteriorly than the p.o.a. In order to determine when the far portion of the spinning stops and the near portion starts, one must observe the larva crossing to the opposite side of the p.o.a. to initiate a strand (if spinning from the left it will reach to the right side of the p.o.a. and initiate a strand there, and then pull it, over the back, to the left anterior again) and terminate the strand at the anterior of the original side.
0) More than 1. When individuals of a species consistently spin more ‘far’ than ‘near’ the ratio will be more than 1.

1) Less than 1. When individuals of a species consistently spin more ‘near’ than ‘far’ the ratio will be less than 1

?) Not applicable. Species which do not spin the initial structure longitudinally, do not have this character.

4) Pupation tent:

The pupation tent is the most anterior portion of the cocoon, and is built predominantly during the initial structure stage. It extends beyond the collar of the cocoon when the cocoon is completely built.

0) Rudimentary. The pupation tent is minimal.

1) Complete. A complete pupation tent using many longitudinal strands is built. Many species continue to build onto the pupation tent during the ‘S’ cross stage as well.

5) S stage:

The second stage of spinning (For more information on this stage, see section 5.1.2.2).

0) Major. If more than 40% of all crosses made during the spinning process are S crosses, then this stage is considered to be the major spinning stage.

1) Minor. If this stage is approximately equal to the other stages, in terms of number of crosses, then it is considered to be a minor stage in the spinning process.

6) S stage spinning:
0) Walls and roof. In the spinning of the S stage, the strands of silk placed to solidify the entire visible cocoon. These species build not only on the walls of the cocoon, but also on the roof. The spinning on each of these locations is shared approximately equally during this stage.

1) Walls. The individuals of these species build predominantly on the walls of the cocoon. Although some silk is also placed on the roof, it is minor compared to the concentration on the walls.

7) Back cross stage:

This is the third stage of spinning for most species (for more information on this stage, see section 5.1.2.3).

0) Absent. The individuals never perform a back cross.

1) Present. The back cross occurs when the larva crosses over the back from the apex of the cocoon to the front on the opposite side. The head of the larva does not extend outside the mouth of the cocoon after the initiation of the back cross stage.

8) Area of concentration during back stage:

0) All. The larva spins on the roof, wall and floor of the cocoon, in approximately equal proportions during this stage.

1) Walls. The larva concentrates almost totally on the walls of the cocoon. Although it does spin some silk on the roof and floor of the cocoon, the spinning on these two areas is very minor compared to the spinning performed on the walls of the cocoon.
7) Not applicable. Individuals of these species do not spin the back cross stage.

9) Intermediate back and S stage:

   0) Present. In some species, the individuals do not initiate the back cross stage completely, but rather, perform some back crosses, then revert back to the S cross stage, before spinning back crosses irreversibly (not necessarily present in all individuals of a species).

   1) Absent. Species where all of the individuals complete the S cross stage and then begin the back cross stage, with no intermediate stage at all.

   ?) Not applicable. Individuals of these species do not spin the back cross stage.

10) Wall-floor connection:

See the description of the cocoon spinning behaviour of S. vittatum (section 5.1.2.3) for further explanation of this character

   0) Absent.

   1) Present. Some species make a visible connection between the wall of the cocoon and the substrate upon which they are spinning.

11) Roof construction:

   0) S stage. In these species the majority of the roof of the cocoon is built during the S cross stage.
1) Back cross and pull front stages. The individuals of some species start the construction of the roof during the end of the back cross stage.

2) Pull front stage. The individuals of these species do not start the roof construction early, and only build it during the pull front stage.

12) Collar:

0) Absent. The individuals of these species do not build for an extended period at the anterior portion of the cocoon.

1) Present. The larva will spin for an extended time, usually about 10 seconds at the anterior end of the future cocoon. The larva will stay on one side of the collar predominantly, during each cross.

13) Collar construction:

0) Predominantly pull front stage. The collar of the cocoon is spun predominantly in the pull front stage.

1) Back cross and pull front stages. Individuals of some species start to build the collar during the back cross stage and continue to build it throughout the pull front stage.

2) S cross stage. One species, that builds a boot shaped cocoon, builds the collar during the S cross stage.

?) Not applicable. Individuals do not perform the pull front stage.

14) Pull front stage:
During this stage the major focus for spinning is the roof of the cocoon. This is the stage where the majority of work on the collar of the cocoon is performed. It is also the stage where any anterodorsal projections are built. (For more information concerning this stage see description of cocoon spinning behaviour for *S. vittatum*. (Section 5.1.2.4))

15) Anterodorsal projection:

Individuals of some species will pull strands to the medial anterodorsal portion of the cocoon and instead of stopping at the collar will pull slightly further to produce an anterodorsal projection. They will gradually add to the projection, during successive pull front crosses, until it is complete.

0) Absent.
1) Present.

16) Projection construction:

0) All. The individuals of the species spin onto the projection each time a pull front cross is made.

1) Most. These individuals do not spin onto the projection until a few crosses have been completed, after which they spin onto the projection during each cross.

?) Not applicable. Individuals of these species do not construct a projection.
17) Projection spinning:

0) Longitudinal. The projection is constructed by an individual pulling longitudinal strands past the collar of the cocoon. It will gradually add on to this projection, but only with longitudinal strands. The projection made is usually square or rectangular in appearance.

1) Longitudinal and transverse. The extension is initiated in the same fashion. However, when adding to the projection, the larva will rotate onto the collar. This makes the projection into a ‘V’ shape with the sides of the projection reinforced to a similar degree as the collar.

?) Not applicable. The individuals do not spin a projection.

18) Pull stage:

During this stage the major focus of the spinning is the floor of the cocoon, especially in the posterior section of the cocoon. (For more information concerning this stage see description of cocoon spinning behaviour for S. vittatum. (Section 5.1.2.5)).

0) Absent. Individuals of these species do not perform this stage.

1) Present. For individuals of these species the floor of the cocoon is the area of spinning concentration during this stage.

19) Inner cocoon:

Some species, especially ones that have more “circular” cocoons, have an inner cocoon that is spun inside the exterior wall of the cocoon (Figure 11). This inner cocoon is the same shape as the pupa.
Figure 11: Schematic diagram of the inner cocoon found in some species of black flies.
water flow

posterior

outer cocoon

inner cocoon

collar

anterior
Therefore, after the pupa wriggles back into the cocoon, it is held tightly in the cocoon while metamorphosing into an adult.

0) Absent.

1) Present.

(The flip cross stage is the last stage of spinning and it is found in all species of black flies studied, therefore it is a sympleisiotypic character. The posterior floor of the cocoon is spun during this stage.)

20) U turn direction:

When the larva releases its posterior circlet of hooks it turns itself into a 'U' shape with the anterior and posterior ends of the larva sticking out of the finished cocoon.

0) Horizontal. Individuals are always parallel to the surface on which they spun.

1) Predominantly horizontal. Most of the individuals (greater than 50%) U turn horizontally, but some individuals U turn perpendicular to the spinning surface.

2) Predominantly vertical. Most of the individuals (greater than 50%) U turn perpendicular to the spinning surface, but some individuals will U turn horizontally.

21) Leaves gaps (windows) in the walls of the cocoon

During the back cross stage the larva will leave fenestrae in the walls of the cocoon. The larva does this by crossing to one side and building at the anterior portion of the cocoon, as normal for an
Figure 12: Phylogeny of the black flies produced using 23 characters for 20 taxa representing 6 genera. Characters are optimized on the tree using the respective numbers from section 5.1.1. Character numbers followed by a (a) or (b) represent the stage of the transformation series. The C.I. is 0.91 and the R.I. is 0.96. (For sample sizes see Table 2)
individual that performs the back cross stage (character 7). When spinning the wall, however, it moves posteriorly a slight distance from the collar before laying down silk, thereby leaving a gap in the wall.

0) Absent
1) Present
?) Not applicable

22) Ratio of duration of back cross stage compared to the ratio of the duration of the pull cross stage (See Appendix 1, Figure 1-10, stage 3 vs. 5)

0) Not significantly greater.
1) Significantly greater.
?) Unknown

23) Ratio of number of S crosses + number of back crosses: number of pull front crosses + number of reinforcement crosses (See Appendix 1, Figure 1-10, stage 8+9/10+12).

0) Less than 1.70
1) Greater than 1.70
?) Unknown

5.4 RESULTS OF THE PHYLOGENETIC TREE RECONSTRUCTED

The tree produced, using both quantitative and qualitative characters, is one that was constructed using 23 characters for 20 species of black flies. Twenty of the characters are strictly qualitative in nature and the remaining three (characters 3, 22 and 23) are
quantitative. The tree has a consistency index of 0.91 and a retention index of 0.96 (Figure 12).

The codes were derived by watching as many individuals of each species as possible. In four of the species studied, there are only 1 or 2 individuals recorded. In these situations, if the cocoon that was spun by any individual corresponded to the descriptions of the cocoons for that species in the literature, that individual was considered to be representative of the species.

*Prosimulium* was used as the outgroup because it is the most ancestral genus in this analysis based on morphological information (Currie 1986). *Parasimulium* is the most ancestral genus of black flies, but this genus is only found in northwestern United States and therefore, could not be collected for this study. *Parasimulium* spin cocoons that are loose sacs, similar to *Prosimulium mixtum*, which were used in this study. Outside of Simuliidae, some Chironomidae species also spin silk, but it was also not possible to video tape any of individuals of these species.

Note: In this description, the presence of an (a) or (b) after the character number represents the stage of the transformation series (Figure 12).

5.4.1 Characters supporting Node 1 (Figure 12)

It is impossible, from the phylogenetic tree produced from these data to determine relationships between *Prosimulium* and *Cnephia dacotensis* (Figure 12). This is likely because only two observations of *Cn. dacotensis* were made. This species has an irregular cocoon compared to other members of that particular genus. *Prosimulium* is
being used as the outgroup for the analysis, therefore the first separation indicates the outgroup.

5.4.2 Characters supporting Node 2 (Figure 12)

*Stegopterna mutata* (Malloch), *Simulium* and *Eusimulium* are separated from *Prosimulium* and *Cn. dacotensis*. This is supported by three characters, including the presence of the back cross stage (7), the presence of an intermediate stage between the S and back stages (9) and the ability of some individuals to U turn vertically (20a).

5.4.3 Characters supporting Node 3 (Figure 12)

*Stegopterna* is the sister group for the *Simulium + Eusimulium*. This is supported by 12 characters.

The cocoon foundation (1) for the three previous genera was reliant on having two surfaces at some angle to each other (in this case, 90 degrees between the wall and floor of the flume). Therefore, the *Cnephia, Prosimulium* and *Stegopterna* need a crack or corner in order to successfully spin their cocoons. This is not the case, however, for the *Simulium + Eusimulium* where individuals in these genera can spin in a corner or crack, but can also spin in the open, using only one surface.

The type of front strands (2) laid down are also different. The *Simulium + Eusimulium* place strands longitudinally, whereas the three previous genera all place front strands transversely.

The area of concentration during initial structure (far/near ratio) (3a). This character is a transformation series with the character that supports this node being when there is more spinning at the posterior of the cocoon, or far crosses, than there are at the anterior of the cocoon, or near crosses.
The pupation tent (4) is built in two different ways. The three ancestral genera all spin the pupation tent by laying transverse strands anteriorly to the future cocoon. This method yields a pupation tent that is attached to the entire opening at the anterior end of the cocoon. The *Simulium* + *Eusimulium* species spin the pupation tent during the near portion of the front cross stage and also during the early crosses of the S cross stage. These strands get laid down only on the lateral surfaces of the future cocoon. The pupation tent appears only as weak extensions of the lateral surfaces when the cocoon is completed.

*Prosimulium, Cnephia* and *Stegopterna* all have the S stage (5) as the major stage of spinning (i.e., over 40% of crosses). In contrast, all species in the clade *Simulium* + *Eusimulium* have reduced the dependence on the S stage and use it to get inside the initial structure, and to initiate the wall structure. However, *S. pictipes*, which has a boot-shaped cocoon, builds the raised portion of its cocoon during this stage as well.

In terms of what area of the cocoon gets built during the S cross stage (6), the *Prosimulium, Cnephia* and *Stegopterna* use this stage to spin the walls and the roof of the cocoon. In contrast, the *Simulium* + *Eusimulium* spin predominantly on the walls of the cocoon.

There is a shift in regard to the area of concentration during the spinning of the back cross stage (8). *Stegopterna mutata* has a back cross stage, but the method of spinning is different. *St. mutata* spins predominantly on the floor of the cocoon, and only performs this stage for a brief period of time. In contrast, *Simulium* + *Eusimulium* spin
predominantly on the walls of the cocoon, with some strands placed on
the roof of the cocoon as well, during this stage.

The wall to floor connection (10) character, which is quite visible
in a number of species separates the Simulium + Eusimulium from the
other genera. None of the species observed from the other three
genera make a strong connection between the foundation surface and
the wall of the cocoon which is being built. All the Simulium +
Eusimulium make some form of a connection, but it is stronger in
some species than in others. It would be possible to make the
strength of this connection, a character, but excellent recordings are
required for each species.

There are two characters relating to the collar of the cocoon. The
primitive genera do not spin a collar at all (12). Therefore, it is only
present in Simulium + Eusimulium. For those species that have a
collar, it is either built during the pull front stage, or during the back
cross and pull front stages. At this node the symplesiotypy is the
former character state, that is, the collar being built in the pull front
stage (13a).

The pull front (14) and the pull stage (18) are not found in any
of the primitive genera, but are both found in all the Simulium +
Eusimulium. The roof is generally the major focus during the pull
front stage, but is also where the collar and projections get built as
well. During the pull stage the larva concentrates on spinning onto the
walls and floor of the cocoon.

These characters, 12 in all, support the monophyly of Simulium+
Eusimulium. The tree then splits into two clades: the Eusimulium and
the Simulium. The Eusimulium group in this study includes: Eus.

5.4.4 Characters supporting Node 4 (Figure 12)

The first character is the presence of an inner cocoon (19). The inner cocoon is only built by species in the *Eusimulium* complex and it is built, for most species, during the pull stage and completed in the reinforcement stage. *Eus. rivuli* is the only exception; because individuals have an extremely abbreviated pull stage, almost the entire inner cocoon is built during the reinforcement stage.

The second character is the fact an individual will only spin on the roof of its cocoon during the pull front stage (11a). It is interesting to see the amount of completion of the cocoon at the beginning of this stage. There is a pupation tent and very strong walls of the cocoon, indicating the entire shape of the future cocoon; however, it is possible to see through the roof of the cocoon, right to the foundation surface. At the completion of this stage, the entire roof is filled.

The third character is not quite as definitive a character in support of this group. This character is the predominance of individuals to U turn vertically as oppose to horizontally (20b). This is a slightly noisy character because there are reversions back to the predominantly horizontal state in two of the taxa. These are *Eus anatinum/congareenarum* and *Eus. gouldingi*. To code this character all the individuals were studied and if more than half of the individuals in a particular species were U turning vertically they were coded as 2, and if more than half were horizontal, they were coded as 1. There were extremely small sample sizes for both *Eus.*
anatinum/congareenarum and Eus. gouldingi, therefore, they were scored with a '?' until more data can be obtained.

5.4.5 Characters supporting Node 5 (Figure 12)

The next monophyletic group in the tree is that of Eus. craigi, Eus. caledonense, Eus. croxtoni, Eus. gouldingi, Eus. anatinum/congareenarum and Eus. rivuli with Eus. euryadminiculum as the sister species to this group. This node is supported by three characters, all of which involve the anterodorsal projection. The first is merely the presence of the projection itself (15). All the species in this group build a projection at the anterior end of the cocoon, on the dorsal surface.

The two other characters concern the type of spinning involved (17a) and the duration of spinning (16a) of the projection. The duration is determined by the number of crosses in the pull front stage, where there is spinning onto the projection. This particular node is supported by the individuals that build onto the projection for most, but not all of the crosses performed during this stage. Typically the first one or two crosses are performed without starting to build the projection.

The type of spinning (17a) is actually the manner in which the projection gets made. This character state involves the individuals pulling back to the anterior of the cocoon and extending past slightly to initiate the projection. Then the individual moves posteriorly until it reaches the collar on the spinning side. When the collar is reinforced the larva will also reinforce the projection. This tends to give the projection a triangular appearance, with thick sides.

5.4.6 Characters supporting Node 6 (Figure 12)
This node is supported by the collar construction character (13b). The construction of the collar, ancestrally, occurs in only the pull front stage. However, at this node the construction of the collar occurs initially at the end of the back cross stage and continues through the entire pull front stage.

5.4.7 Characters supporting Node 7 (Figure 12)

This is supported by the type of spinning made on the anterodorsal projection (17b). These species all remove the rotation from the process. This means that when a pull front cross is made, the larva will extend past the collar slightly. Each successive cross will extend the projection further from the collar. However, the individual does not rotate; therefore it does not reinforce the sides of the projection or really connect the projection to the collar in any way. These species have square, or rectangular shaped projections.

5.4.8 Characters supporting Node 8 (Figure 12)

There are two characters which support this node. The first is the duration of projection construction (16b). This character is the number of crosses spent building onto the projection during the pull front stage. The ancestral state, as mentioned previously, was that individuals spun onto the projection in most but not all the crosses. From this node, however, the individuals of these species spin onto the projection in all the crosses during this stage.

The second character is the far/near ratio (3b). These three species have more near than far front crosses during the initial structure stage.

5.4.9 Characters supporting Node 9 (Figure 12)
When considering the monophyly of the *Simulium*, things are not quite as clear. *S. decorum* Walker can be moved to be the ancestral taxa for the *Eusimulium* without the C.I. or R.I. changing. This is because there are not many characters that support the placement of this species. Another problem with *S. decorum* is that it is an anomalous species within the *Simulium*, when considering its cocoon. *S. decorum* has an extremely reduced cocoon, which may make it problematic when determining relationships using the cocoon spinning behaviour only. This species will be placed as the ancestral taxa for the *Simulium* based on other morphological and cytological information (Figure 15).

There are two characters which support the monophyly of the *Simulium*. One is when the roof gets constructed during the spinning process (11b). In the *Simulium* the roof is constructed in both the back cross stage and the pull front stage. The other is a quantitative character indicating the relative importance of the back cross stage in the spinning process (22). All species of *Simulium* (as well as *Eus. rivuli*) have a significantly longer proportion of time spent in the back cross stage than the pull stage.

5.4.10 Characters supporting Node 10 (Figure 12)

The initiation of the collar construction beginning in the back cross stage (13b) is one of the characters that clusters the two *S. tuberosum* populations, *S. rostratum*, *S. venustum/truncatum*, *S. krebsorum*, *S. dixiense*, *S. pictipes* and *S. vittatum* with *S. decorum* as the sister group. The other is the ratio of the number of ‘S’ and back crosses compared to pull front and flip crosses (23). This gives the appearance of a clustering of the first 3 stages and the last 3 stages.
with the former significantly greater than the latter in most of these 5 taxa (Appendix 2, Figures 1-10, stages 6-9 vs. 10-12)

5.4.11 Character supporting Node 11 (Figure 12)

The far/near ratio (3b) is the character in support of this node.

5.4.12 Character supporting Node 12 (Figure 12)

The intermediate S and back stage (9) character supports this node. In all other species individuals will sometimes perform a number of S crosses after the back stage has started. These S and back crosses are considered to be an intermediate phase. However, an intermediate stage was not observed in any of the individuals (see Table 2) of the two *S. tuberosum* populations. If more individuals were observed, this intermediate stage may be present in some individuals of *S. tuberosum*. Regardless, it is apparently extremely rare in this species, if present at all.

5.4.13 Character supporting Node 13 (Figure 12)

Character 21, leaving gaps in the walls of the cocoon, is only found in these two taxa. These two species both leave fenestrae in the structure of the cocoon during the back cross stage.

6.0 DISCUSSION

6.1 DISCUSSION OF THE COCOON SPINNING BEHAVIOUR OF *Simulium vittatum*

The cocoon spinning behaviour of *S. vittatum* is an intricate and elaborate process. Researchers in the 1920-1960's had problems describing the behaviour accurately and completely with only the naked eye or binocular microscope (Tonnoir 1923; Peterson 1956; Burton 1966). Certain aspects of the behaviour are visible under these conditions, while others are too complex and require the ability to watch the stages many times over. The video playback system
allowed for counting crosses, timing stages and close observation of
the spinning behaviour during each stage.

6.1.1 Pre Cocoon Spinning Behaviour

The larva must find a suitable location (depending on the
species) and then it must clean the area thoroughly. The pupa will be
inside the cocoon for a number of days before it ecloses as an adult
(Peterson 1981) and, therefore, the cocoon must remain properly
anchored during this time. The cleaning behaviour is visible with the
naked eye and all previous researchers noticed this behaviour. Burton
(1966) stated that the larva is checking the substratum, while others
correctly stated that the behaviour was both to check and clean the
area prior to spinning (Wu 1931; Peterson 1956; Crosskey 1990).

Wu (1931) described the cleaning behaviour of the larva as: “it
bends back and applies its mouthparts to a certain point on the
support near its posterior end, then suddenly pulls its head away with
some force”. This corresponds to the tugging, described previously;
however, no one described the scraping which comprises most of the
cleaning time. Wu (1931) stated that the cleaning may last longer
than one minute. In reality, the larva can clean for more than 45
minutes. Previous researchers might have thought that the scraping
was some stage of spinning. During the cleaning stage, crosses similar
to the ‘front’ cross stage (section 5.1.2.1) of spinning are seen and the
larva spends a similar amount of time on each side before crossing
over, as in the later stages of spinning. Because of the lighting used
during recording, it is possible to see individual strands of silk when
they are present. During the cleaning stages there are no signs of any
strands of silk being laid down. The larva concentrates its cleaning
upstream of the p.o.a., and therefore the area where the future cocoon is located is free of debris before the spinning begins.

6.1.2 Spinning Behaviour

The actual cocoon spinning behaviour is much more difficult to see and descriptions of this behaviour are very diverse. Peterson (1956) is the only author who studied *S. vittatum*. Peterson states that the order of cocoon spinning is: an initial framework, a net-like structure, the spinning of the cocoon starting with the collar, the apex of the cocoon, the top and finally the floor of the cocoon. There are a number of problems with this. Peterson (1956) states that the larva lays down a framework by placing pads of silk to the front, back and on both sides of itself and then connecting these pads with strands of silk. This has not been apparent in any of the individuals studied. *S. vittatum* does lay down one or two pads of silk in front of itself but the framework is not apparent. The net-like structure is likely a pupation tent (Crosskey 1990). This structure is believed to keep the pupa from being swept away in the current when it breaks out of its larval skin and wriggles back into the cocoon. The pupation tent is built during the first two stages of cocoon spinning in *S. vittatum*. Other important parts of the cocoon are also built during this time. This structure, therefore, is not built before the cocoon is spun as described by Peterson (1956). Peterson states that the next structure to be built is the collar; however, it is impossible for the larva to build the collar without the initial structure, walls and part of the top of the cocoon already in place.

Aside from the problems occurring with the description of the behaviour, the largest problem is that the description of the actual
movements of the larva are missing. For example, when describing the building of the top of the cocoon Peterson (1956) states that “the larva twisted and turned, spinning silk from side to side and forward and backward soon giving shape to the cocoon”. This description makes it impossible to understand what the larva is actually doing throughout the spinning process.

The only author to determine the stages properly was Tonnoir (1923) studying S. tillyardi. He describes the first two stages quite well describing both the ‘front’ (Tonnoir 1923, p. 166, Figure 1) and ‘S’ crosses (Tonnoir 1923, p. 168, Figure 4). When the larva stops using the ‘S’ cross and starts to cross over its back, Tonnoir found it difficult to see exactly what it was doing and stated that the “larva continues to spin the inside of the cocoon in a random or irregular fashion”. He did notice that the concentration was on the floor of the cocoon at the end of spinning. Again, however, the description of the movements of the larva are not descriptive enough to use as character states for phylogenetic analysis of the behaviour.

Other researchers were less accurate in their descriptions (see Puri 1925; Wu 1931; Burton 1966). There are two major points upon which the authors cannot agree. One is whether the larva detaches itself during the spinning process. In the present study no larva of any species were observed detaching until immediately before it breaks the larval skin. Most of the researchers also stated this (Tonnoir 1923; Wu 1931; Peterson 1956; Crosskey 1990), while others stated that it does detach and reattach itself during the cocoon spinning process (Puri 1925; Burton 1966). They also disagree on the sequence of spinning, in particular, whether the floor is built first (Wu
6.1.3 Post Cocoon Spinning Behaviour

After the cocoon has been spun, the larva must remove the larval skin and secure itself inside the cocoon. This behaviour is visible, even with the naked eye and all researchers who described this behaviour, described it properly (see descriptions by Tonnoir 1923; Wu 1931; Burton 1966; Crosskey 1990).

The duration of the entire behaviour was recorded by a few researchers. Wu (1931) stated that it took 25 minutes for the post cocoon spinning behaviour, and 50-60 minutes for the spinning, totaling 75-85 minutes for the entire process. Puri (1925) and Crosskey (1990) both stated that the whole process from the initiation of spinning to the removal of the larval skin takes 75-90 minutes. In the current study the entire process for *S. vittatum* took 37-52 minutes. Previous researchers, however, were studying different species. It is possible that this large discrepancy in spinning time is a result of previous authors inadvertently including time that the larva spent cleaning the substrate with the time it was actually spinning the cocoon.

When comparing these descriptions to the other species in this study, it is clear that the descriptions are not sufficient. The duration of spinning time does vary from species to species. However, the basic pattern of spinning behaviour is the same for all *Eusimulium* + *Simulium*. Therefore, although different species of *Simulium* were being scrutinized, it is highly unlikely that the descriptions any of
Figure 13: Phylogeny produced using the gap-coding technique (Goldman 1988) for the quantitative data, using 13 characters for 10 taxa. The C.I. is 0.49 and the R.I. is 0.46.
Figure 14: Phylogeny produced by comparing means and confidence levels. There are 10 taxa and 30 characters used in this analysis. The C.I. is 0.54 and the R.I. is 0.47.
Figure 15: Phylogeny reconstructed for the species of black flies under investigation in this study, using morphological and cytological information (Cytological information from: Hunter 1990, Hunter and Connolly 1986, Rothfels 1979, Rothfels and Golini 1983, Rothfels et al. 1978 and Dr. P. Adler, Clemson University, pers. comm. 1995. Morphological information from: Dr. D.C. Currie, University of Toronto, pers. comm. 1995).
these authors gave sufficiently describe the cocoon spinning behaviour.

**6.2 COMPARISON OF BEHAVIOURAL PHYLOGENY TO MORPHOLOGICAL AND CYTOLOGICAL PHYLOGENY**

In this examination no outgroup was available for Simuliidae, therefore, following Currie (University of Toronto, pers. comm. 1995), *Prosimulium* will be taken as the outgroup for the rest of the taxa.

The 23 characters used to construct this tree produces a phylogeny of the black flies that closely resembles phylogenies composed using other characters (see Figure 15). The *Simulium* are clustered, the *Eusimulium* are also clustered and the monophyly of *Simulium + Eusimulium* is supported by a number of characters. The C.I. of 0.91, the R.I. of 0.96 and the fact that this tree could not be generated randomly all support the view that this is a valid tree which represents the evolution of black flies. The only concern with the phylogenetic analysis occurs with the number of equally parsimonious trees. There are 18 trees that all have the same length. The major differences between these trees, however, are the placement of *S. decorum* and *S. vittatum* as either ancestral to the *Simulium* or to the *Eusimulium*, and the trichotomy of *Eus. craigi*, *Eus. caledonense* and *Eus. croxtoni*. Of these trees, the one that most closely resembles the phylogeny of the black flies constructed using morphological and cytological information is shown in Figure 12.

**6.3 COMPARISON OF THE QUANTITATIVE TREES TO EXISTING PHYLOGENIES**

Both of the trees produced using completely quantitative information yielded phylogenetic trees that were not even remotely
similar to the phylogenies in existence for the black flies (Figures 13 and 14 vs. 15).

The tree produced using the gap coding technique (Figure 13) for continuous data produced one tree with a C.I. of 0.49 and a R.I. of 0.46. However, when analyzing this tree, there are a number of noticeable problems. All of the 13 characters on the tree are very difficult to trace, having reversions or convergences present. It is also apparent that the clustering of species into monophyletic groups does not place individuals of the same genus together, and even separates the two populations of *S. tuberosum* into separate monophyletic groups. This phylogeny was yielded little or no useful phylogenetic information and was not used in the overall analysis.

The second method of quantitative analysis also produced a phylogenetic tree that did not resemble the previously established tree. There were four equally parsimonious trees produced, having a C.I. of 0.53 and a R.I. of 0.47. These low numbers indicate that there is a considerable amount of convergence or reversion found in this phylogenetic analysis. Due to these characters having little or no evolutionary significance, they were not included in the overall analysis.

However, when analyzing the data in using the means and confidence intervals, two trends were noted in the data that closely resembled the trends found in the existing phylogeny (Appendix 1, Figures 1-10). These characters were added to the qualitative phylogeny as characters 22 and 23 (see section 5.3). The three species that were recorded from South Carolina, *S. pictipes*, *S. krebsorum* and *S. dixiense*, were analyzed after these characters were added to the
phylogeny. All three fit the two trends, indicating that these two characters are valid for the phylogeny.

It is obvious from the results that quantifying behaviour is easy, yet getting useful phylogenetic information from this data, is much more difficult. Part of this results from the continuous nature of the data which makes it difficult to code as discrete data. Also, the means are placed in order from smallest to largest when coding; however, sometimes the means can be close together, but be given different codes, depending on where the standard deviation falls. Other times two means that are very distant may get the same code.

What basically seems to be the case with the cocoon spinning behaviour though, is that differing amounts of the ratio of total spinning time and crosses may be spent on a particular stage, but observable differences in the cocoon shape or form may not occur. Therefore, there is both individual variation and species variation. The individual variation leads to high standard deviation and the species variation indicates that two species, even though closely related will not necessarily share very many characters, yet the end product may look very similar. Another problem with studying the ratios of stages and crosses is that if one stage is significantly different in two different species, then another stage must also be different to compensate in the ratio procedure.

Therefore, it is believed that in the case of black fly cocoon spinning, and possibly many other behaviours, that it is important to concentrate on the actual intricate movements and timing of aspects of the particular behaviour being analyzed. The idea of quantifying behaviour may be, as in this case, not worth the time and effort that
inevitably is necessary. It is especially important to realize that behavioural information could be a lot less tedious if the emphasis was on understanding the qualitative information found in the behaviour.

6.4 Ectemnia invenusta

6.4.1 Placement of Ectemnia invenusta in the phylogenetic tree

Unfortunately, the entire cocoon spinning of *E. invenusta* has not been recorded. It is extremely vulnerable to temperature increases and although a few have successfully spun in the flume, none were recorded. The only recordings that have been made are of individuals that only have spun until the back cross stage and then have incurred some problems. It is interesting to note, however, that considering the characters that can be assessed during these first three stages (6 in all, see Appendix 2, Table 3) that *E. invenusta* shares all of these characters with the *Eusimulium + Simulium* clade.

These characters are: the cocoon foundation being one surface (1), the type of front strands being longitudinal (2), the presence of a pupation tent (4), the S stage spinning on the walls (6), the back cross stage present (7) and the spinning on the walls of the cocoon during the back cross stage (8). From these characters it would seem that *E. invenusta* should be placed in the phylogeny as an unresolved trichotomy with *Simulium* and *Eusimulium* (Figure 12). However, until the entire behaviour is analyzed, it will be impossible to ensure that this is the appropriate placement for this taxon.

6.4.2 Discussion of the stalk building behaviour in Ectemnia invenusta

There is only casual observation of *E. invenusta* as preserved specimens, and no attempt in the literature to describe either the
behaviour of stalk building or cocoon spinning. When *E. invenusta* stalks were described (Wolfe and Peterson 1959) there was mention of particles of sand and dirt found in the stalk. It appears that these likely stick to the stalk, and then when the larva reinforces the entire stalk, the particles get trapped. Whether this is done purposely to give the stalk rigidity, or not, is unknown.

6.5 POSSIBLE EVOLUTIONARY SIGNIFICANCE OF CHARACTERS

Many of the characters used to reconstruct the phylogeny of the black flies can be analyzed from not only the stand-point of phylogenetic information of the character, but also the possible evolutionary significance of how or why each character may have evolved. In this portion of the discussion the characters will be assessed in order, and those having a plausible evolutionary explanation will be discussed.

1) Cocoon foundation:

The transition between requiring two surfaces to successfully spin a cocoon, to only requiring one surface would have allowed the species requiring one surface, to inhabit a greater range of stream types with a greater range of substrates, flow types and flow speeds.

2) Type of front strands:

It is possible that the transition between using transverse strands for the initial structure to using longitudinal strands actually allowed for the movement away from cracks and out to the open area of a substrate to spin the cocoon. The species which need the cracks or corners spin transversely to the flow of water and therefore to the larva's body direction. In doing so, the larva connects the two
surfaces with the strands of silk. The individuals that can spin in the open place the strands of silk parallel or longitudinally. Using itself as the support necessary to start the cocoon, the larva can build a free-standing cocoon. The strands of silk are initiated posteriorly to the p.o.a. and are then drawn around the individual’s dorsal surface before being terminated anteriorly. Thus, the dorsal surface of the individual keeps the silk off the substrate and allows the cocoon to be built using only one surface for the foundation.

3) Area of concentration during the initial structure stage (far/near ratio)

The situation of having a far/near ratio of greater than one, seems to be common in species that build more circular cocoons, or at least have horizontally expanded cocoons, like *Eus. gouldingi*, although this is not always the case. This could be explained for the species that do spin more horizontally expanded cocoons, as it is the initial structure which determines the future shape. Theoretically, since during the far portion of this stage the initial structure of the future posterior of the cocoon gets built, an individual that builds an expanded cocoon should place more emphasis on this portion of the spinning process.

The species that have the near portion of the initial structure stage greater than the far portion, tend to have more streamlined cocoons with elaborate anterior portions of the cocoon. The *Eusimulium* that exhibit this character have strong pupation tents, collars and anterodorsal projections, while the *Simulium* which display this character have pupation tents and strong collars. It is believed that it is more important for the individuals of species with greater
near portion spinning during the front stage to construct a solid framework at the front of the cocoon, as a support structure for these additional characters.

4) Pupation tent:

It is apparent that the pupation tent is an important structure for all species of black fly that were studied (n=21). It is found in all species regardless of the method of spinning during the initial structure stage.

5) S stage:

The S stage of spinning appears to be the most difficult for the larva to perform. On many occasions, in a variety of species, an individual would break the initial structure and have to begin the entire process again with the front cross stage. At times, individuals also found it difficult to get inside the initial structure, and would have to make a number of attempts to do so. Therefore, reducing both the number and proportion (found in *Eusimulium* and *Simulium*) of the S crosses would likely be an evolutionary modification not only to spin the cocoon more quickly, but also more efficiently.

7) Back stage:

The presence of the back cross stage allows the individual to place less structural pressure on the cocoon. The S cross stage, which is the dominant stage for *Prosimulium*, *Cnephia* and *Stegopterna*, places stress on the initial structure of the cocoon. Reducing the proportion of the S crosses (although minor reduction for *Stegopterna*) must aid in reducing this stress. It is probable, as well, that more silk can be laid down in a shorter period using back crosses, because there is constant spinning. In the S cross, each time the individual reaches
the front of the cocoon, the spinning stops while it ducks its head under to get to the other side.

10) Wall-floor connection:

It is likely that this structure allowed species to not only move to a one surface foundation, but also to be able to tolerate faster flowing streams and rivers, as well as fluctuating flow in their locations, without the pupa getting dislodged from the substrate. The pleisotypic state of this character is the absence of the connection (*Prosimulium, Stegopterna* and *Cnephia*).

11) Roof construction:

It is believed that it is more important to build the walls of the cocoon before the roof, which is why the walls were built at the beginning of the cocoon spinning process. When the back cross stage was added to the black fly behavioural repertoire, the spinning during the S stage was then concentrated to predominantly spin the walls of the cocoon. The roof could be delayed, and was initiated in the back cross stage. Also, when performing a back cross the larva spins on the roof of the cocoon in all instances, because it pulls across the roof of the cocoon to get to the other side. It may be that the walls are structurally more valuable to the cocoon than the roof, or that they are merely necessary in order to build the roof successfully.

In *Eusimulium*, however, the roof only gets built in the pull front stage. When the larva pulls to cross to the other side during the back cross stage the larva starts at the apex of the cocoon and pulls down the wall of the opposite side. Therefore, the larva does not really cross on the roof at all. It is possible that the *Eusimulium* do not need to start the roof construction in the back cross stage, because they
spend a large portion of time performing the pull front stage. Many of the *Eusimulium* species produce an anterodorsal projection during the pull front stage which takes some extra time. They must continue this stage until the projection is completed, and during that time the roof of the cocoon can be entirely constructed.

12) Collar:

The addition of a collar, whether thick or thin is presumably to add structure to the cocoon, to help the cocoon hold its shape and not collapse on the pupal respiratory filaments.

13) Collar concentration:

The species that have the collar built solely in the pull front stage, obviously have collars, but they are not very strong. All the species that initiate the collar spinning process in the back cross stage have well defined collars. Initiating the collar construction in the back cross stage gives a greater number of crosses where silk can be added to the collar.

*S. pictipes* builds a boot-shaped cocoon. A weak collar is built on the opening of the cocoon during the ‘S’ cross stage. This indicates that a collar is important to the cocoon structure. It would be impossible for *S. pictipes* to build the collar when other species, that build slipper shaped cocoons, build their collar, due to the nature of the structure of the cocoon.

14) Anterodorsal projection:

It is believed that the projection aids in the flow of water over the respiratory filaments of the pupa (Dr. D. Craig, University of Alberta, pers. comm., 1994). As mentioned previously, it may also aid in the removal of the larval skin. Ancestral species, that have no
projection, have the ability to U turn vertically. The species with
projections, however, tend to use the vertical U turn more often. The
process of U turning requires energy, so presumably any aid to
remove the skin would likely be used.

16) Projection construction:

The species that build onto the projection during each of the pull
front crosses probably need to do this in order to construct larger,
more elaborate projections. These individuals therefore, spin onto the
projection as many times as possible.

19) Inner cocoon:

This structure appears to be necessary for species that build a
cocoon that is wider than their body. If the cocoon is much wider than
the body of the pupa, the pupa will not be held tightly in the cocoon
during pupation. It is most likely that the widening of the cocoon
occurred, and then a method of reducing the movement of the pupa
inside the cocoon evolved. The other possibility is that this inner
cocoon evolved to keep bacteria and other small organisms from
getting in between the pupa and the cocoon walls (Dr. D. Craig,
University of Alberta, pers. comm., 1995) and once an inner cocoon
was made, for what ever reason, this freed the individuals to expand
their cocoon.

The former explanation seems more likely, but it is impossible to
determine from this study. It seems more likely because, in the
process of spinning, the widening of the cocoon is initiated in the
initial structure stage and must be produced prior to the building of
the inner cocoon which is spun in the pull stage. Thus, the sequence of
evolution is likely that the cocoon was widened first, to aid in flow
over the respiratory filaments and that this triggered the individuals to have to build a structure to keep the body of the pupa from being loose inside the cocoon. Some species have much wider cocoons than others, but in all species of *Eusimulium*, an inner cocoon is visible when a good recording is made.

20) U turn:

Regardless of the species, the ability to U turn vertically may be advantageous to increase the speed and pressure with which the larva can push against the cocoon. This may increase the efficiency of removal of the larval skin. Thus, having the option of U turning horizontally or vertically, may allow the individual to choose the alignment which allows for the most pressure to remove the larval skin.

In species that build an anterodorsal projection, U turning vertically allows more of the body surface to be pushed against the cocoon. Thus, the dorsal surface of the larva is bent backwards and is touching itself and the ventral surfaces are touching the substrate on the floor and the projection on the anterodorsal surface of the cocoon. It seems possible that this added friction aids in the removal of the larval skin.

21) Leaves gaps (windows) in the walls of the cocoon.

The description of cocoon shapes in relation to the flow of water over the respiratory filaments, has been discussed by Eymann (1991). He did not discuss how the presence of fenestrae affects the water flow. It seems likely, however, that these windows are left in the walls of the cocoon to allow for improved flow over the respiratory
filaments (Dr. D. Craig, University of Alberta, pers. comm. 1995 and Dr. P. Adler, Clemson University, pers. comm. 1995).

6.6 BEHAVIOURAL PHYLOGENETICS

The outcome of this thesis indicates that there certainly is very useful phylogenetic information found in behavioural characters. However, there have been a number of papers questioning the validity of using behavioural characters for phylogenetic reconstruction (Atz 1970, Urbani 1989, Carpenter 1987). These authors hold that behavioural information should be used to understand evolution, as opposed to characterizing major evolutionary trends. They conclude that morphology is much more conservative than behaviour, and therefore morphology should be the basis of phylogenetic reconstruction.

Due to the results of this thesis, it appears that the conclusions of these authors may be false, and there are two major reasons for this discrepancy. The first is that authors tend to include all behavioural characters without putting them through a rigorous analysis, as is done with morphology. For example, in a very large number of species, ranging from such diverse groups as: vertebrates, birds, reptiles, amphibians, and insects (both aquatic and terrestrial), nocturnal species are present. It has long been discounted as a character in most cladistic analyses, because it is subject to convergent evolution, yet it is still used as a ‘behavioural’ character in some insect phylogenies (see Carpenter 1987). Morphologists tend to select characters that are known, or at least believed, not to be homoplastic. However, when these same authors select ‘behavioural’ characters, they do not assess each character for its **validity**. Using these
characters to state that behaviour is more plastic than morphology, is questionable.

The second problem, is that often the 'behavioural' characters used in a phylogenetic analysis are not behaviour at all, but rather, the end product of a behaviour. Some examples of this are: spider webs, caddisfly cases, and prey capture in vespids. These end products may in fact have some very useful phylogenetic information, but until the behaviour is thoroughly analyzed to ensure that there is not a large amount of convergence in these characters they should be used with caution. Authors would find that they would get more useful characters if the actual behaviour were analyzed, broken down into its constituent parts and those assigned character status.

In the example of prey capture, Carpenter (1987) uses as a character, live vs. dead prey. It seems likely that this could make erroneous groupings if used, because this character could be subject to convergent evolution. It would seem more informative if one assessed how the prey was captured and assign character status to that aspect of the behaviour. As mentioned earlier, when studying the building of a spider's web (Coddington 1990), one should be analyzing the behaviour of building the web, as opposed to the web itself.

It seems that often times a very complex behaviour will be reduced to one character, which often does not give useful phylogenetic information. If more time were spent in the selection of the behaviours to be studied and the analysis of the behaviour it is likely that these analyses would yield additional characters for phylogenetic analysis.

7.0 CONCLUSIONS
Most systematists feel that the more useful characters there are for a particular analysis, the better off the resulting phylogeny will be. In this regard, behavioural data has been largely overlooked in the past for a variety of reasons. Behaviour is sometimes technically difficult to capture (i.e., with video, or the naked eye) in enough detail to allow for the assessment of character status. Also, some believe that behaviour is more labile than morphology and therefore not useful phylogenetically.

I believe that this thesis indicates that behavioural characters are valid for use in phylogenetic reconstruction, if used properly. The behaviour selected should be one that is complex and not subject to rampant homoplasy. One should also avoid selecting the end product of the behaviour until the actual behaviour itself has been thoroughly analyzed. The technical problems with behaviour are being overcome rapidly.

When a truly accurate phylogeny is being reconstructed for any organism, all potentially informative characters should be used. If a behaviour exists with the above mentioned characteristics, these characters should be included in the analysis.

As shown in this thesis, the analysis of the behaviour of black fly cocoon spinning closely resembles previous phylogenies reconstructed using morphological and cytological information. Therefore, these 23 characters would be useful phylogenetic characters to create an even more robust phylogeny for the black flies.
8.0 LITERATURE CITED


9.0 Appendix 1: Data matrices
Table 1: The data matrix for the 20 species used to reconstruct the phylogeny found in Figures 12.
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Table 2: The data matrix for the phylogeny found in Figure 13. Characters were coded using the gap-coding technique for continuous data.
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Table 3: The data matrix for the phylogeny found in Figure 14. Characters were coded by comparing means and confidence intervals of one stage to all the other stages.
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Appendix 2: Means and confidence intervals for the six stages (duration and cross data for each) for thirteen species.
Figure 1-10: The means and 95% confidence intervals of the ratio of duration of the stage/total time spent spinning (stage: 1) initial structure, 2) ‘S’ cross, 3) walls and back cross, 4) pull front, 5) pull, 6) reinforcement) and the ratio of the number of crosses/total number of crosses (stage: 7) front cross, 8) ‘S’ cross, 9) back cross, 10) pull front cross, 11) pull cross, 12) flip cross) for each species.

Figure 1: *Simulium venustum/ truncatum*
Figure 2: *S. rostratum*
S. rostratum

percent vs. stage
Figure 3: *S. tuberosum*
Figure 4: *S. tuberosum* (population collected in Alberta)
S. tuberosum (Alberta)
Figure 5: *S. krebsorum*
Figure 6: *S. dixiense*
Figure 7: *S. vittatum*
Figure 8: *S. pictipes*
Figure 9: *S. decorum*
Figure 10: *Eusimulium euryadminiculum*
Figure 11: *Eus. craigi*
Figure 12: *Eus. croxtoni*
Figure 13: *Eus. rivuli*